BILIRUBIN RETINOPATHY*†

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BILIRUBIN may enter into the eye in jaundiced patients from the blood, or it can be elaborated locally from intra-ocular haemorrhages ("posthaemorrhagic xanthochromia", Fuchs, 1919; Appelmans and Michiels, 1951). The former occurrence is characterized by a much lower bilirubin level (50–220 fold) in the ocular media (aqueous, vitreous), than in the serum (Naumann and Young, 1960; Toews and Basu, 1962). Investigating the distinctive features of posthaemorrhagic xanthochromia, we found an exclusively indirect-reacting (unesterified) form and higher concentrations of bilirubin in the eyes than in the serum of affected patients.

The prevailing conditions (relatively high concentrations of unesterified bilirubin in an extravascular fluid of low albumin-content (vitreous) in contact with ganglion cell-rich nerve tissue (retina)) are identical with those postulated by Blanc and Johnson (1959) as determining factors in the pathogenesis of bilirubin-encephalopathy of infants (kernicterus). According to these authors, bilirubin itself is the toxic agent, and is capable of entering into and interfering with the metabolism of ganglion cells. This view was challenged by Haymaker, Margoles, Pentschew, Jacob, Lindenberg, Arroyo, Stochdorph, and Stowens (1961), who emphasized in kernicterus the role of liver damage inhibiting the selective permeability and energy utilization in the central nervous system. According to this view, intracellular bilirubin is not the cause but a mere indication of cell necrosis.

Stimulated by this controversy, we investigated the following problems:

- (1) Does bilirubin interfere with retinal functions in posthaemorrhagic xanthochromia and in experimentally-induced analogous states?
- (2) If it does, how can it enter into the cells?
- (3) Once it has entered, what are its metabolic effects?

The first problem was approached most conveniently by electroretinography, as changes of visual acuity may also have extraretinal causes.

The classical observation of Claireaux, Cole, and Lathe (1953) of a cerebral lipid fraction with bilirubin-retaining capacity, and the recent evidence of exclusion or binding of certain substances by the sialic acid end-groups of the cell surface (Woolley and Gommi, 1964; Kraemer, 1966; Brunngraber and Brown, 1967; Brunngraber, Dekirmenjian, and Brown, 1967) suggested the bilirubin-receptor role of the sialic acid-containing lipids (gangliosides). This aspect of bilirubin-binding was tested by lipid thin-layer chromatography of retinæ incubated with bilirubin.

As regards the third problem, Waters and Bowen (1955) and Day (1956) described the reduction of respiration, and Biesold, Liebold and Theile (1962), the uncoupling of oxidative phosphorylation of brain slides and homogenates, respectively, by bilirubin. In the case of the retina, we measured these effects *in vitro* without slicing or homogenizing.

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Methods

(1) To investigate the features of posthaemorrhagic xanthochromia, the eyes of patients with a history or signs of previous intra-ocular bleedings were watched for the appearance of a greenish-yellow discoloration of the anterior segment and of the vitreous structures. Ten eyes without perception of light had to be removed, and samples of aqueous and/or of the vitreous body were obtained from these enucleated eyes for bilirubin determinations. From two further eyes without perception of light, and from one eye with 0.06 visual acuity, 0.15 ml. samples of aqueous were withdrawn by aid of an Amsler cannula and subjected to analysis. Simultaneously fasting blood samples were taken for bilirubin determinations. Indirect- and direct-reacting bilirubin was measured by the van den Bergh reaction according to the method of Jendrassik and Gróf (1938). One aqueous and one vitreous sample very rich in bilirubin (Cases 1 and 9, Table) were diazotized and extracted with butanol, and chromatography was performed according to the method of Schmid (1957); simultaneously crystalline bilirubin and bile (containing bilirubin glucuronide) were treated in the same way.

(2) Electroretinography was performed according to the procedure of Karpe (1948a) using Electoretinograph M 12 B (Elema), and single flash stimuli yielding 120 lux at the eye of the patient. As these ERG alterations may be due to various pathological changes causing haemorrhages, or to haemosiderosis consequent upon haemorrhages (Karpe, 1948b) as well as to bilirubin toxicity, experiments were performed on rabbits to elicit identical ERG alterations by the juxta-retinal injection of erythrocytes or bilirubin, respectively, to exclude these complicating factors.

From the ear vein of five albino rabbits (1 to 2 kg.) blood was withdrawn in sterile heparinized tubes and the erythrocytes were washed three times with saline.

Left Eyes.—After anaesthesia with pantocaine and retrobulbar procaine, 0.2 ml. aqueous was withdrawn from the anterior chambers, and 0.1 ml. packed autologous erythrocytes was injected through 20-gauge needles perforating the sclerae in a bevelled position parallel to and 8 mm. behind the limbus. By aid of a sclera lamp, semicircular bands of injected erythrocytes became visible behind and partly in front of the retinae. Xanthochromia of the anterior segment appeared on the 4th postoperative day and increased during the following week.

Right Eyes.—15 days later 0.1 ml. of 50 mg. per cent. bilirubin solution was injected by the same technique into the right eyes. The solution was made by dissolving 5 mg. crystalline bilirubin in 0.5 ml. warm 0.1 N sodium carbonate and diluting aliquots of it with 20 volumes of sterile serum from the same animals.

For electroretinography, the rabbits were rolled in towels; after instillation of pantocaine, a hypodermic needle was introduced through the conjunctiva laterally behind the globe, and 0.5 ml. procaine having been injected through it, the needle was left in place as a reference electrode. Two thin copper wire sutures were led along the procaine-infiltrated lid margins serving as lid retractors and earth electrodes. Finally, the corneal contact lens electrode was inserted, and after 10 minutes' dark adaptation, the records were taken using 480 lux stimuli.

(3) To investigate the bilirubin-binding lipid fractions, cattle retinae were cut into 9 to 16 mm.² pieces not more than 2 hours *post mortem*, and 5 g. quantities were incubated for 20 minutes at 37°C. with occasional stirring in 6 ml. of the following medium:

2.5 ml. Krebs-Ringer solution supplemented with 1 per cent. bovine albumin + 2.5 ml. 0.1 N Na₂HPO₄ in which 12.5 mg. bilirubin were dissolved with the aid of 2 drops of 2 M KOH + 0.010 g. Na₂ATP dissolved in 1 ml. water. The 208 mg. per cent. bilirubin-containing medium remained clear in spite of a pH of 7.4. A further 5 g. retina were incubated in a medium differing from the former only in that it contained no bilirubin.

After incubation, the retinae were washed three times in saline; extraction of lipids according to the method of Bloor (Entenman, 1957, *proc.* 1) yielded the richest extract in all lipid fractions. On

the other hand, the extraction procedure as suggested by Suzuki (1965) was helpful in the isolation of a high yield of gangliosides obtained by shaking the chloroform-methanol extracts of retinae with 0.88 per cent. KCl solutions and with water, leaving all other lipids in the organic phase.

Lipid fractions of the extracts were separated by ascending chromatography on 250 μ activated Kieselgel G-/Merck/-layers, developed by the following solvent systems:

System 1.—For the separation of different polar lipids

(a) chloroform-methanol-water-ammonium hydroxide (64 : 27 : 4 : 0.1 lower phase),

(b) chloroform-methanol-water (65 : 25 : 4).

System 2.—For the separation of different gangliosides, n-butanol-pyridine-water (3 : 2 : 1).

System 3.—For the separation of apolar from polar lipid fractions, the double solvent system of Sachs and Wolfman (1964).

For identification of the fractions, the following sprays were used:

For bilirubin, the diazo reagent as described by Jendrassik and Gróf (1938).

For demonstration of phospholipids, the molybdenum blue reagent of Zinzadze (Dittmer and Lester, 1964).

For gangliosides, Bial's orcinol reaction (Honegger, 1962).

As general lipid stains, bromthymol blue (Jatzkewitz and Mehl, 1960) or oxytetracycline (Kahán, 1967).

Reference substances: lecithin and cephalins prepared from brain; brain-ganglioside (Svennerholm, 1956), consisting mostly of the monosialoganglioside GM₁ (Svennerholm, 1964); crystalline bilirubin (Reanal).

(4) Some metabolic effects of bilirubin on the retina were investigated *in vitro*. Changes in respiration were measured by the Warburg manometric method, and the simultaneous inorganic phosphate-uptake by the retinae was determined by phosphorus analysis of the medium. One 100 mg. strip of freshly-removed cattle retinae (not later than 2 hours *post mortem*) was immersed in the main compartment of each Warburg vessel containing the following media:

2.6 ml. Krebs-Ringer-solution fortified with 30 mM glucose, 20 mM succinic acid, and 20 mM glutaminic acid (neutralized), 2 mM disodium salt of adenosine triphosphate + 0.3 ml. 0.1 M Na₂HPO₄ brought to pH 7.6 with 2 M KOH (two drops of the latter to 5 ml. of the former are needed), in which before addition to the fortified Krebs-Ringer-solution 500, 250 or 125 mg. per cent. bilirubin were dissolved.

The central wells contained 0.2 ml. 2 M KOH soaked in a filter paper-ring, the side-arms 0.5 ml. 50 per cent. trichloroacetic acid.

Before attaching the vessels to the manometers, 0.05 ml. saturated yeast-hexokinase in 1 per cent. glucose prepared according to the method of Berger, Slein, Colowick, and Cori (1946) was measured into the main compartments. A pair of vessels contained the same bilirubin concentrations (8, 4, and 2 M $\times 10^{-4}$), a pair of flasks with retina and the same solutions contained no bilirubin, and a further pair of vessels contained the same solutions without bilirubin and the retina was substituted by 0.1 ml. saline (thermobarometer). Oxygen consumption was measured at 30°C. at 10 minute intervals for 30 minutes (gas atmosphere: air) after 5 minutes' thermo-equilibration. The reaction was stopped by addition of the trichloroacetic acid from the side arm, except in the thermobarometer in the vessel of which a 100 mg. strip of retina was immersed and likewise fixed immediately with the trichloroacetic acid. Experiments were carried out in triplicate. Oxygen consumption was calculated from the means of the first and second 10 minutes' incubation data. Inorganic phosphate determinations were performed according to the method of Baginski and Zak (1960) from 0.1 ml. supernatants of the TCA fixed, ice-cold, and centrifuged vessel contents. Phosphate-uptakes were calculated by subtracting the phosphorous values of the 0, 2, 4, and 8 M $\times 10^{-4}$ bilirubin-containing vessels from those of the thermobarometers. Each mean of phosphate-uptakes and of oxygen consumptions at the different bilirubin levels was expressed in μ -atoms per

1 mg. retina (dry weight), per hour (standard deviation: 10–18 per cent.). As measures of oxidative phosphorylation at different bilirubin concentrations, the above values of phosphate-uptake were divided by the corresponding oxygen consumption (P/O ratio).

Results

(1) Features of Posthaemorrhagic Xanthochromia

Yellow discoloration of the anterior chamber and/or of the vitreous body was recognized in fourteen cases. From their data (Table) the following points may be emphasized:

TABLE
CLINICAL AND LABORATORY DATA OF CASES EXHIBITING BILIRUBIN FORMATION IN THE EYES
FROM INTRA-OCULAR HAEMORRHAGES

Case No.	Age (yrs)	Causes of Haemorrhages			Site of Haemorrhages		Fate of Eye		Days between Haemorrhages and Appearance of Bilirubin	Indirect Reacting Bilirubin (mg. per cent.) in		
		Injury	Surgery	Proliferative Retinopathy	Pre-retinal	Retro-retinal	No Light-Perception	Re-moved		Aqueous	Vitreous	Serum
1	83	+			+	+	+		12	14.0		
2	51	+				+	+	+	17		3.8	
3	49	+				+	+	+	38	1.3		0.3
4	79		+			+	V: 0.4 → 1.0		7			
5	37		+			+	V: 0.06 → 0.5		6	0.7		
6	61		+		+		+	+	30		0.7	
7	2			+		+	+		730	4.0		
8	18			+		+	+	+	150	3.8		0.9
9	76			+	+		+	+	?		5.3	1.3
10	57			+	+	+	+	+	365	3.0	3.1	0.5
11	56	+	+	+		+	+	+	7	6.8		0.9
12	84	+		+	+		+	+	?	3.9		1.6
13	8	+		+		+	+	+	90	3.0		
14	70	+		+	+		+	+	10		2.2	1.0

(a) By clinical examination, and in ten cases by dissection of the enucleated eyes, the haemorrhages were found in one or both of two sites: in the retrovitreal space, or between the choroid and the detached pigment epithelium. From the latter site, the yellow pigment may appear much later in the anterior chamber.

(b) In the aqueous, the average bilirubin concentration was 4.5 mg. per cent. In the vitreous the average was 3 mg. per cent. Both the aqueous and vitreous body values were always higher than the serum levels of the same patients.

(c) In both aqueous and vitreous samples, bilirubin was invariably of the indirect reacting type, and proved to be chromatographically identical with unesterified bilirubin (Fig. 1, overleaf).

(2) Functional Damage of the Retina caused by Bilirubin

(a) *Posthaemorrhagic xanthochromia of patients.*—In twelve eyes of fourteen patients under study light perception faded, and when ten eyes were removed and dissected, the

FIG. 1.—Paper chromatogram of extracts made according to Schmid (1957) of:
(a) diazotized xanthochromic aqueous humour, Case 1.
(b) bile.
(c) crystalline bilirubin.

Note: (b) and (c) are reference substances of bilirubin glucuronide and unesterified bilirubin, respectively.

retinae were found to be stained yellow by bilirubin. Certain case histories argue even more in favour of bilirubin toxicity:

Light perception vanished simultaneously with the appearance of heavy xanthochromia consequent on haemorrhagic endogenous uveitis while the fellow eye, suffering from a more severe analogous process but without xanthochromia, recovered (Case 8).

Visual acuity, which decreased simultaneously with the appearance of xanthochromia complicating cyclodialysis performed in cases of open-angle glaucoma (Cases 5 and 4), improved to the original value as xanthochromia disappeared.

The ERG appeared to be a sensitive and unequivocal indicator of retinal damage coinciding with xanthochromia.

In the last-mentioned, Case 4, electroretinography was performed at the peak and during the spontaneous disappearance of xanthochromia following cyclodialysis. The ERG of the xanthochromic right eye with normal disc and 0.4 visual acuity was subnormal ($b = 100 \mu V$), while that of the left eye with cupped disc, incipient cataract, and 0.1 visual acuity was normal ($b = 300 \mu V$). With the disappearance of xanthochromia, the ERG of the right eye gradually attained a normal course (Fig. 2) and the visual acuity improved to 1.0.

FIG. 2.—Electroretinograms of Case 4:

- (a) Left eye (cupped disc).
- (b) Right eye (normal disc) at peak of xanthochromia.
- (c) Right eye, xanthochromia beginning to disappear.
- (d) 3 years later, no xanthochromia. Calibration: $500 \mu V$.

(b) Experimentally-induced ERG-alteration due to bilirubin

(i) At the peak of xanthochromia induced by the injection of erythrocytes (10th postoperative day), the ERG was reduced to a very quick, burst-like, cornea-negative wave of short (40 msec.) latency; 5 days later this gave place to a slower cornea-negative wave in all animals. No *b* waves were perceptible (Fig. 3).

FIG. 3.—Pathological electroretinograms of rabbit No. 59, left eye, induced by pre- and retroretinal injection of washed erythrocytes leading to xanthochromia.

(a) 10th and (b) 15th postoperative day. Normal ERG before injection. Calibration: 500 μ V.

(ii) The effect of injected bilirubin was followed at regular intervals during the first post-operative hour in one rabbit, and 1 hour, 12 hours, and 10 days after the injection of bilirubin in five rabbits. 5 minutes after the injection, the 250 μ V preoperative amplitude of the *b* wave decreased only transiently to 100 μ V, surpassing its pre-operative amplitude in the 60th minute. 12 hours after the administration of bilirubin, the *b* wave became definitely subnormal, with a preserved *a* wave. 10 days after the injection, the *b* wave was almost extinguished in all animals and the *a* wave was somewhat increased (62 μ V) (Fig. 4).

FIG. 4.—Electoretinogram changes in rabbit No. 59, right eye, induced by pre- and retroretinal injection of bilirubin.

(a) before, (b) 5 minutes, (c) 60 minutes, (d) 12 hours, and (e) 10 days after injection. Calibration: 500 μ V.

Thus the following common traits of ERG alterations, both human and experimental, and even those brought about by intra-ocular injection of bilirubin in rabbits may be outlined:

- (a) The decrease or disappearance of *b* waves, with preservation or increase of cornea-negative components, and
- (b) the relatively late onset, slow evolution, and long duration of these changes.

(3) Bilirubin-receptor Role of Gangliosides

The extracts of retinae incubated with bilirubin, obtained by Bloor's procedure, were

intensively yellow-coloured, although unbound bilirubin is almost insoluble in ethanol-ether (3 : 1) mixture and ether.

The chromatogram, developed in the System 1a and sprayed with diazo reagent, proved to contain a slowly-moving fraction carrying all the bilirubin differing from the behaviour of unesterified bilirubin standard which remained at the start line. The site of the bilirubin-carrying fraction in this system did not correspond to those of phospholipids of retinal extracts demonstrated by the phosphormolybdenum blue reaction, but did however correspond to the place of gangliosides in this solvent system. The ganglioside-nature of the bilirubin-carrying fraction is even more evident on the chromatogram developed in System 2: a spot of high R_f value, yellow coloured before spraying and marked by a dotted-line contour, proved to correspond to the site of monosialogangliosides of retinal extracts and to that of the ganglioside reference substance, as seen by Bial's orcinol reaction (Fig. 5 a-c, opposite). Conversely, the bilirubin standard remained at the start line in this system also.

Extraction and partition of lipids according to the method of Suzuki (1965), when applied to retinæ incubated with bilirubin, yielded coloured KCl-H₂O-washings, pointing to the ganglioside-bound state of its bilirubin content. The organic phase also remained faintly yellow, however. When subjected to TCL in System 2, in the washings originating from retinæ incubated with bilirubin, four bilirubin-carrying fractions were found and marked by dotted-line contours, corresponding in site to the four ganglioside fractions obtained by this procedure as seen by Bial's reaction. Only traces of gangliosides remained in the organic phase; however a heavy spot of unbound bilirubin was found at the starting line in the latter (Fig. 5d). Conversely, when developed in System 1b and demonstrated by the molybdenum blue reaction, most of the phospholipids were found in the organic phase which did not coincide with the site of bilirubin, mostly remaining at the starting line in this phase as did the bilirubin standard (Fig. 5e). Nor did the bilirubin-carrying fractions coincide with the sites of cholesterol or triglycerides, as visible on the chromatograms developed in System 3 and demonstrated by bromthymol blue. These fractions are also in the organic phase. In this system unbound bilirubin is migrating and bound-bilirubin is retained at a lower site corresponding to that of gangliosides (Fig. 5f).

All chromatographic evidence points to the affinity of unesterified bilirubin to the gangliosides of the retina. Its binding to the latter may be the primary event when retina contacts bilirubin-containing media.

(4) Some Effects of Bilirubin on Retinal Metabolism

The respiration of retinæ is depressed by only 6 per cent. in the presence of $2 \text{ M} \times 10^{-4}$ bilirubin. Respiration decreased with increasing bilirubin concentrations, being as low as 11 per cent. of retinal respiration found in the absence of bilirubin, when incubated in the medium containing $8 \text{ M} \times 10^{-4}$ bilirubin. Conversely, phosphate uptake fell from the bilirubin-free value (2.35μ atoms P/1 mg. retina, dry weight/1 hour) to 2.5 per cent. of the former in the presence of $2 \text{ M} \times 10^{-4}$ bilirubin, and to 0.25 per cent. in $8 \text{ M} \times 10^{-4}$ bilirubin, a concentration found in ocular fluids of clinical cases (Fig. 6, opposite).

The results correspond to an uncoupling of oxidative phosphorylation with a simultaneous depression of oxidations, these effects taking place presumably at the level of the mitochondria.

In the frozen sections of retinæ shaken for 35 minutes in the Warburg vessels containing $8 \text{ M} \times 10^{-4}$ bilirubin for metabolic studies, the bulk of bilirubin was found in the ganglion

FIG. 5.—Thin-layer chromatograms of lipid extracts. *a-c* prepared according to Bloor from retinæ incubated with bilirubin (R_b) or without bilirubin (R_w). *d-f* prepared according to Suzuki; O: organic, A: aqueous phase. Reference substances: L: lecithin (containing also some sphingomyelin and lysolecithin); K: cephalins; G: ganglioside GM_1 ; B: crystalline bilirubin dissolved in $CHCl_3$. Solvent systems (see Text): 1*a* in *a* and *b*; 1*b* in *e*; 2 in *c* and *d*; 3 in *f*. Identification reactions: diazo reagent in *a*; molybdenum blue reaction in *b* and *e*; bromthymol blue in *f*; Bial's orcinol reaction in *c* and *d* (the bilirubin-containing yellow fractions were marked by dotted contours before spraying). The Figure demonstrates in retinal extracts, and particularly in the aqueous phase of the latter, the migration of bilirubin with the gangliosides.

FIG. 6.—Effect of different concentrations of bilirubin on respiration and oxidative phosphorylation of bovine retinæ shaken in Warburg vessels, supported by succinate + glutamate and glucose + hexokinase.

cell layer (Fig. 7); some bilirubin staining may also be detected in the ellipsoid region of the photoreceptors.

FIG. 7.—Frozen section of bovine retina shaken in a Warburg vessel at 30°C. for 30 minutes in the presence of $8 \text{ M} \times 10^{-4}$ bilirubin. Haematoxylin stain. $\times 100$.

Discussion

From the available data, the following chain of events may be traced:

(1) From intra-ocular haemorrhages contiguous with the vitreous surface and/or the uvea, both rich in cells with reticulo-endothelial function (*e.g.* the hyalocytes: Szirmai and Balazs, 1958), bilirubin can be formed, causing xanthochromia. Intra-ocular origin is proven by the invariably indirect-reacting, unesterified nature of the pigment, and by its relatively high and always higher concentration in the aqueous and vitreous body than in the serum of the same patients, a situation which is never found when bilirubin is derived from jaundiced plasma (Naumann and Young, 1960; Toews and Basu, 1962).

(2) When high concentrations of unesterified bilirubin, even in the presence of albumin, touch the retina, the pigment is selectively bound to the gangliosides of the latter, these being constituents of cell surface. In this context it should be remembered that, in frozen sections of kernicteric brains, the primary event is the alteration of the cell surface, and increased refringence of the latter (Blanc and Johnson, 1959).

(3) The next step is the uncoupling of oxidative phosphorylation leading to an energy-deficit of the cells involved. This effect occurs presumably at the level of mitochondria. As this effect is immediate, it can be localized only in layers adjacent to the surface, in the ganglion cells where the bulk of bilirubin is found. The layer of ellipsoids, one site of the oxidative phosphorylation of the retina, may be involved.

(4) According to these considerations, the bilirubin elaborated from intra-ocular haemorrhages should be able to inflict immediate damage upon the retina; this may either have no effect upon the ERG, as the ganglion cell layer has no role in the generation of ERG-potentials (Haschke and Sickel, 1962), or may be manifested in the decrease of cornea-negative potentials if the ellipsoids are involved. Against expectation, ERG alterations due to bilirubin were found; however, they appeared with delay and were manifested in the decrease or disappearance of the cornea-positive potentials, and in the preservation or increase of the negative components. All these controversies may be explained only by an interneuronal spread of bilirubin from the sites of maximal concentrations into the bipolars, the site of maximal toxicity.

The occurrence of bilirubin retinopathy in patients free from any hepatic disease lends support to the view of Blanc and Johnson (1959) that bilirubin can enter and damage

healthy ganglion cells, especially if it can be shown that the gangliosides of the cerebral nuclear areas have the same affinity for bilirubin.

Summary

The effects of intra-ocular haemorrhage have been studied in man and experimentally in rabbits. It was found that high concentrations of unesterified bilirubin were elaborated, resulting in yellow discoloration of the aqueous and vitreous body with simultaneous deterioration of retinal functions, and characteristic ERG signs.

In lipid extracts from cattle retinae incubated with bilirubin, the latter is selectively bound to the gangliosides. Oxidative phosphorylation of surviving cattle retinae was found to be uncoupled by bilirubin.

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