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Bilirubin kernicterus
ganglioside mitochondria

Bilirubin Interaction with Ganglioside: Possible Mechanism in Kernicterus

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Extract

Reaction of bilirubin with increasing amounts of ganglioside purified from neonatal brain significantly alters the spectral absorption of bilirubin in proportion to the quantity of ganglioside added. Increments in absorbance occur at 353 nm with a prompt but transient increase at 486 nm. A decrease in absorbance occurs which is most marked at 447 nm. When gangliosides are added to bilirubin (9.1 μg/ml or 0.016 μM/ml), the decrease in absorbance is essentially linear up to the highest concentration of purified ganglioside tested (182 μg/ml or 0.097 μM/ml), which represents a molar ratio of 6.1:1. The asymptotic nature of the bilirubin-ganglioside reaction as measured by the decrease in absorbance with time suggests a stoichiometric relationship between the two substances. An isosbestic point was demonstrated at 405 nm. Observations reported here suggest bilirubin reaction with ganglioside is at least a two-step process.

Speculation

Bilirubin cytotoxicity may be related in part to plasma membrane effects which involve bilirubin interaction with ganglioside at concentrations which do not disturb mitochondrial metabolism. The difference between the ganglioside composition of infant and adult gray matter may in part explain the marked cytotoxicity of unconjugated bilirubin for the infant nervous system.

Kernicterus, a bilirubin encephalopathy, results from the accumulation of nonconjugated, non-albumin bound bilirubin which leads to well described changes in the nervous system (6). In most experiments, anoxia (1, 9, 18), or hypoglycemia (17), in association with hyperbilirubinemia, produces more profound lesions in the experimental model (1, 9, 18) than nonanoxic hyperbilirubinemia. Neurons in such lesions demonstrate cytoplasm with myelin figures and dense bodies thought to be a pigment-lipid complex (10). Cytoplasmic membranous bodies have been described in enlarged Purkinje cell mitochondria of Gunn rats although none were present in astrocytes or oligodendrogliocytes (19). The enhanced susceptibility of specific regions of the nervous system to bilirubin toxicity as well as the increased susceptibility of the infantile nervous tissue has not been explained adequately.

Metabolic studies suggest that bilirubin pigment exerts four effects in mitochondrial reactions: (*1*) stimulation or inhibition of respiration depending on concentration of bilirubin, (*2*) abolition of respiratory control, (*3*) uncoupling of oxidative phosphorylation, and (*4*) induction of energy-requiring swelling. Levels of total bilirubin in the range of 10–40 μmol/liter increase oxygen consumption of mitochondria (2, 11). Concentrations necessary to initiate uncoupling of oxidative phosphorylation within adult or infant rat liver and brain mitochondria *in vitro* are much higher than those found in the brains of adult Gunn rats with experimental bilirubin encephalopathy (3, 5). Furthermore, brain mitochondria of newborn guinea pigs with the clinical features of severe bilirubin encephalopathy fail to demonstrate uncoupling of oxidative phosphorylation (4). Bilirubin inhibition of oxygen uptake is greater for whole brain homogenate from newborn rats than from adult rats (21). Mitochondria from whole brain or cerebellum of newborn guinea pigs with bilirubin encephalopathy fail to exhibit

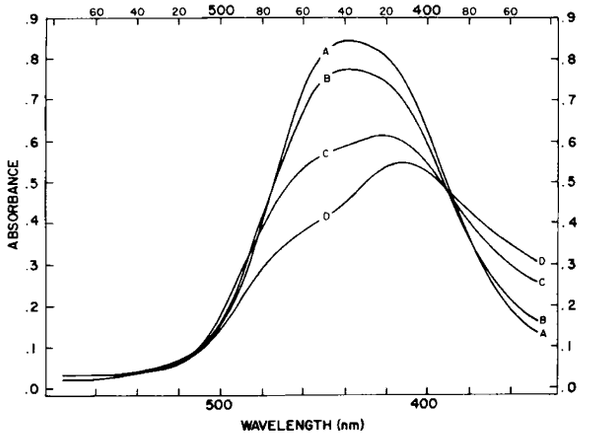

Fig. 1. Absorbance spectrum for bilirubin and various concentrations of ganglioside in aqueous 0.2 M borate buffer with 1.0 mg/ml ascorbic acid. Reaction time was 60 min. Bilirubin was 10 μg/ml (0.017 μM/ml) in all solutions. Ganglioside concentrations were *A:* none; *B:* 31 μg/ml (0.016 μM/ml); *C:* 150 μg/ml (0.080 μM/ml); *D:* 200 μg/ml (0.106 μM/ml).

uncoupling of phosphorylation, and only a small portion of the total pigment in the brain is associated with mitochondria (4).

The ability of bilirubin to bind to protein molecules is clearly established; binding of bilirubin to albumin can be followed by alterations in spectral absorption (14, 15). Bilirubin binds reversibly to lipid; mitochondrial lipid produces two red shifts of absorption from 440 nm to 450 nm and 490 nm (12). This shift can be modified by addition of bovine serum albumin. Kahan *et al.* (8) have reported that bilirubin binds specifically with cerebral gangliosides derived from rat brain. These authors demonstrated chromatographic separation of a complex from brain and spinal cord in cases of kernicterus which demonstrated reactions of both ganglioside and bilirubin. Similar complexes were prepared *in vitro* using rat brain and aqueous bilirubin. This report presents evidence for a two-part interaction between bilirubin and purified ganglioside.

METHODS AND MATERIALS

Ganglioside, purified by the method of O'Brien (13), was obtained from cortical gray matter of two infants; one died within the first 2 weeks of life and the other, a 2,070-g premature infant, died on the third day of life. Purity was ascertained by thin layer chromatography. The predominant gangliosides in the mixture used for the experiments were G_{D1a} (G_3) and G_{D1b} (G_2), an observation which is in agreement with the work of Suzuki (20). Based on the assumption that the sole fatty acid present in the ceramide moeity is stearate (18:0), the molecular weight of the ganglioside was 1,885. Bilirubin from Eastman Chemical Co., purified by the method of Henry (7), was dissolved in 0.2% Na_2CO_3. To minimize degradation, the pH of the solution was adjusted to pH 9.0 with 0.2 M borate buffer containing 1 mg/ml ascorbic acid. Ganglioside was dissolved in 0.2 M borate buffer so that the final reaction mixture was pH 9.0 ± 0.1. All reactions were conducted in very subdued light or total darkness to avoid photodegradation. Spectrophotometric measurements were conducted with a Bausch & Lomb recording spectrophotometer model 505.

Bilirubin solutions were quite unstable at acid or alkaline pH. Sedimentation was noted below pH 8.6 whereas enhanced lability occurred above pH 9.70. For this reason, pH 9.0 was selected for all studies. Photodegradation of bilirubin at this pH was minimal. Ganglioside absorbance was undetectable for the concentrations and spectral range employed.

RESULTS

Bilirubin in 0.2 M borate buffer pH 9.0 has an absorption maximum at 438 nm. Reaction of bilirubin with increasing amounts of ganglioside purified from neonatal brain significantly altered the spectral absorption of bilirubin in proportion to the quantity of ganglioside added (Fig. 1).

Experiments were done under conditions which excluded nearly all light, but photodegradation was still possible because of the light source of the spectrophotometer. Difference spectroscopy, which compensates in part for photodegradation during spectrophotometry, clearly demonstrated alteration in absorption spectrum when the reaction mixture was compared with control bilirubin solution. Progressive change in absorbance occurred with time when a mixture containing 7.2 μg/ml (0.012 μM/ml) bilirubin and 162 μg/ml (0.086 μM/ml) ganglioside was compared with 7.2 μg/ml (0.012 μM/ml) bilirubin (Fig. 2, *top*). Increments in absorbance occurred at 353 nm with a prompt but transient increase at 486 nm. A decrease in absorbance occurs which is most marked at 447 nm. When gangliosides are added to bilirubin (9.1 μg/ml or 0.016 μM/ml), the decrease in absorbance is essentially linear up to the highest concentration of purified ganglioside tested (182 μg/ml or 0.097 μM/ml), which represents a molar ratio of 6.1:1. The asymptotic nature of the bilirubin-ganglioside reaction, as measured by the decrease in absorbance with time (Fig. 2, *bottom*), suggested a stoichiometric relationship between the two substances. An isosbestic point was demonstrated at 405 nm for ganglioside isolated from both brains (Fig. 2, *top*). The insolubility of bilirubin in solutions of pH 8.3 or below precluded experiments at the more acid pH.

DISCUSSION

The development of a transient increase in absorbance at 486 nm corresponds to the wave length of a red shift to 490 nm described by Mustafa *et al.* (12) for the binding of bilirubin with mitochondrial lipids. However, the transient nature of this shift in our

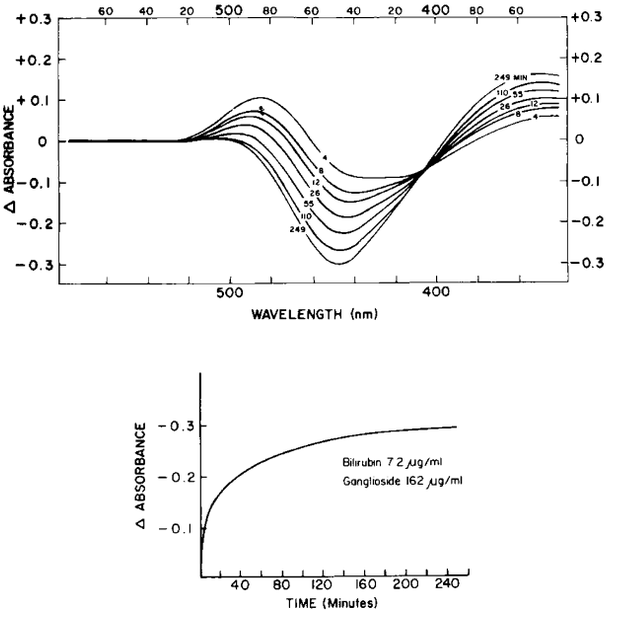

Fig. 2. *Top:* change in absorbance as demonstrated by difference spectroscopy for bilirubin (7.2 μg/ml or 0.012 M/ml) versus bilirubin (7.2 μg/ml or 0.012 μM/ml) plus ganglioside (162 μg/ml or 0.086 μM/ml). Serial recordings at reaction time as indicated (minutes). *Bottom:* decrease of absorbance at 447 nm as a function of time for reaction shown, *top*.

studies, the marked decrease in absorbance at 437 nm which gradually and progressively shifts to 450 nm, and the development of a second absorption peak at 353 nm differ from the reported observations for mitochondrial lipids. Transient increase in absorbance at 486 nm develops promptly and then disappears with time. This observation suggests that bilirubin is bound to gangliosides in two steps. At this point, we are unable to state what the essence of these two steps is, or, in fact, whether they involve bilirubin and gangliosides as such or their degradation products.

Ganglioside occurs in fetal brain even before myelin is present, probably in plasma membranes or other structures. Relatively low levels of ganglioside are found in purified preparations of brain mitochondria, myelin, nuclei, endoplasmic reticulum, and nerve ending particles. Ganglioside composition of whole rat brain changes with age; a relative shift occurring from trisialo- to monosialogangliosides. This shift is much more pronounced in highly purified myelin fractions (21). This difference may be a possible reason for the differential toxicity of bilirubin for newborn brain (6) and brain homogenates (3, 5) in comparison with adult brain preparations. It has been suggested that membranous cytoplasmic bodies, rich in ganglioside, may be masses of plasma membrane (16). In bilirubin encephalopathy, these membranous cytoplasmic bodies may be bilirubin pigment-lipid complexes.

The observations reported here suggest that the bilirubin reaction with ganglioside is at least a two-step process. This reaction, with possible deleterious effect on selected cell plasma membranes, may be one mechanism for bilirubin toxicity. Our studies point to the possibility that gangliosides may alter plasma membranes producing cytotoxicity at tissue concentrations of bilirubin which are too low to uncouple mitochondrial oxidative phosphorylation. In particular, membrane alterations in the dendritic and synaptic regions of neurons may be important in producing cellular dysfunction.

SUMMARY

Spectral absorption is significantly altered when aqueous bilirubin is mixed with purified ganglioside. Difference spectrophotometry discloses a decrease in absorbance at 447 nm with an increase at 353 nm and a prompt but transient increase at 486 nm. An isosbestic point occurs at 405 nm. Interaction, perhaps molecular, between bilirubin and purified ganglioside is demonstrated by these changes for an *in vitro* system. Bilirubin may be toxic *in vivo* to cell elements rich in ganglioside by interference with plasma membrane function. Plasma membrane effects may be crucial at bilirubin concentrations which do not disturb mitochondrial metabolism.

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