BILIRUBIN BINDING TO MYELIN BASIC PROTEIN, HISTONES AND ITS INHIBITION IN VITRO OF CEREBELLAR PROTEIN SYNTHESIS

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SUMMARY: The binding of unconjugated bilirubin to bovine CNS myelin basic protein and to lysine and arginine rich histones has been demonstrated by means of difference spectroscopy and circular dichroism spectroscopy. This is the first demonstration of a brain specific protein that can bind bilirubin and provides a mechanism for bilirubin retention in brain as well as a mechanism for interfering with the normal acidic lipid-basic protein binding interaction. The inhibition of protein synthesis in cerebellar homogenates by bilirubin has also been demonstrated. The inhibition is about 50 per cent in the presence of 100 μM bilirubin and 85 per cent in the presence of 700 μM bilirubin. These results require that the current mechanisms for bilirubin neurotoxicity and cytotoxicity be expanded to include the present findings.

The elevation of serum bilirubin levels in the neonate and the subsequent sequence of events that often, but not always, leads to the accumulation of unconjugated bilirubin in the brain of the newborn is an unsolved puzzle with respect to the mechanism of cyto and neurotoxicity of this bile pigment. The accumulation of unconjugated bilirubin in various tissues is often toxic to the cells that make up the tissue and the metabolic processes necessary for normal functioning. Bilirubin has been reported to have deleterious effects on oxidative phosphorylation (1, 2), DNA synthesis (3), protein synthesis in liver (4), and in tissue culture (2). Silberberg and Schutta (5) reported that bilirubin adversely effects cultures of myelinating rat cerebellum whereas biliverdin or photo-oxidized bilirubin caused no discernable change in these cultures. Also, Schutta and Johnson (6) reported that cerebellar development of the jaundiced Gunn rat was markedly impaired when compared to its non jaundiced litter mates. As a prelude to a study of the developmental biochemistry and morphology of Gunn

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rat cerebellum, a study of bilirubin binding to CNS myelin basic protein and histones was initiated. Also, the effect of exogenous bilirubin on protein synthesis in a rat cerebellar homogenate system was carried out. Although these are model systems, the data that were obtained are relevant to the problem of bilirubin neurotoxicity.

METHODS

Bilirubin (Sigma Chemical Co.) solutions were prepared by dissolving 20 mg. of the solid in 3.0 ml of 0.20 N NaOH. Solubilization was promoted by brief warming in a 50°C water bath with subsequent placement of the flask in the dark for 30 to 50 minutes. After the bilirubin had dissolved the solution was diluted to 10.0 ml with autoclaved, deionized water. Aliquots of this stock solution were added to the incubation mixture to obtain the desired bilirubin concentration.

Difference spectra were obtained with a Beckman ACTA III spectrophotometer.

CD spectra were obtained with a JASCO ORD-CD-5 instrument modified to the SS-20 configuration by Sproul Scientific Instruments.

Myelin basic protein was prepared by a modification of the procedures of Eylar *et al.* (7) and Nakao *et al.* (8) as described by Liebes *et al.* (9).

Lysine and arginine rich histones were purchased from Sigma Chemical Co.

Protein synthesis experiments were done with autoclaved glassware and filter sterilized solutions to exclude bacterial contamination. Experiments were conducted in a darkened room to minimize photo-oxidation of bilirubin.

For each experiment the cerebella from three, eight day old rats (Sprague-Dawley) were removed and placed in 6.0 ml of cold 0.25 M sucrose. The cerebella were homogenized in a Potter-Elvehjem homogenizer. The homogenate that was obtained was used in subsequent experiments without further treatment.

Measurement of the rate of protein synthesis utilized the same protocol as that of Kohl and Sellinger (10). 2.5μ Ci of (4,5-^{3}H)-leucine (New England Nuclear, 42 Ci/m mole) was used per sample. Typically 100 μl of cerebellar homogenate was added to each reaction vessel which in turn contained approximately 6.0 μg

of endogenous RNA. The procedure of Fleck and Begg (11) was used to determine the RNA content of the homogenate.

RESULTS AND DISCUSSION

Figure 1 presents the difference spectrum for the binding of bilirubin to

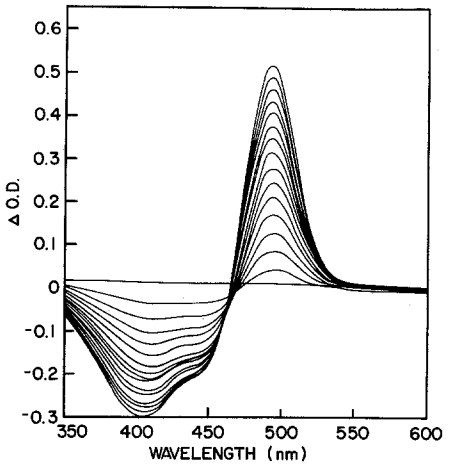

Figure 1 Difference spectra obtained by titration of 27.40 nanomoles of bovine myelin basic protein with incremental additions of 6.99 nanomoles of bilirubin.

myelin basic protein. The number of moles of bilirubin bound per mole of protein cannot be stated with any certainty from this data because of the known preference of myelin basic protein to bind amphipathic molecules via a metachromatic mechanism (9). Nevertheless, the difference spectrum provides the first demonstration of bilirubin binding by a brain specific protein. Corroborative evidence for bilirubin binding is provided by the appearance of an extrinsic positive Cotton effect at 400 nm and a negative Cotton effect at 464 nm (Figure 2). Since unconjugated bilirubin does not exhibit any intrinsic optical activity in the absence of protein, the appearance of this Cotton effect indicates that binding has occurred.

The chemical behavior of myelin basic protein and histones is similar in many respects. Therefore, the binding of bilirubin to lysine rich histone was also studied. The difference spectrum for the binding of bilirubin to histone is shown in Figure 3. Extrinsic Cotton effects were also seen in the CD spectrum.

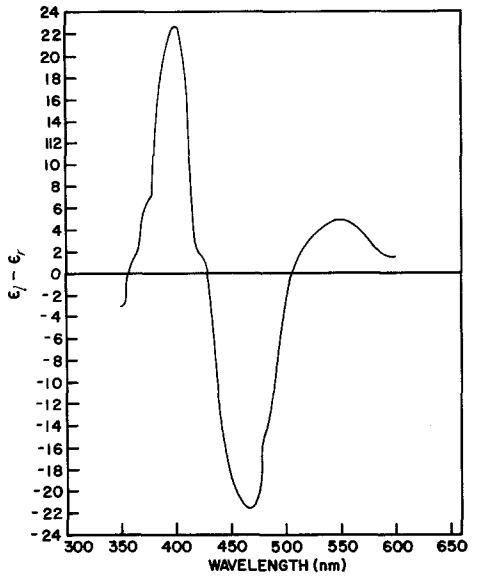

Figure 2 Circular dichroism spectrum of 0.16 micromoles of bilirubin in the presence of 0.22 micromoles of basic protein.

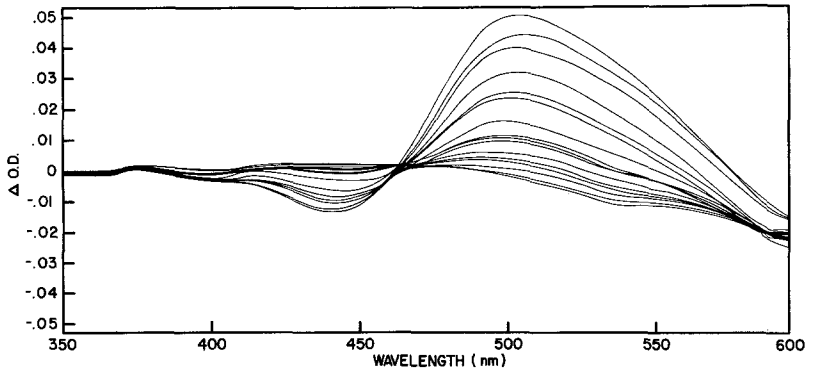

Figure 3 Difference spectra obtained by titration of 5.15 M lysine rich histone with incremental additions of 0.64 micromoles of bilirubin.

The binding of bilirubin to histones led to an examination of the effect of bilirubin on protein synthesis, using the cerebellar homogenate system, the rate of protein synthesis indicated by the incorporation of (4,5-^{3}H)-leucine in the presence and absence of exogenous bilirubin was studied. The data plotted in Figure 4 show that for this system, the rate of leucine incorporation into protein is linear for 30 minutes in the absence of bilirubin. In the presence

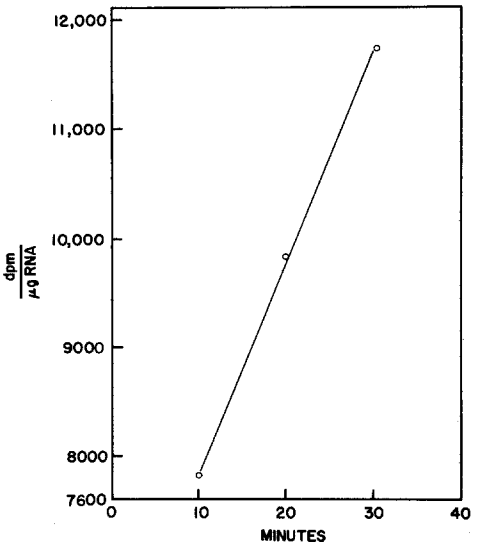

Figure 4 The incorporation of (4,5-^{3}H)-L-leucine as a function of time by rat brain cerebellar homogenate protein synthesizing system.

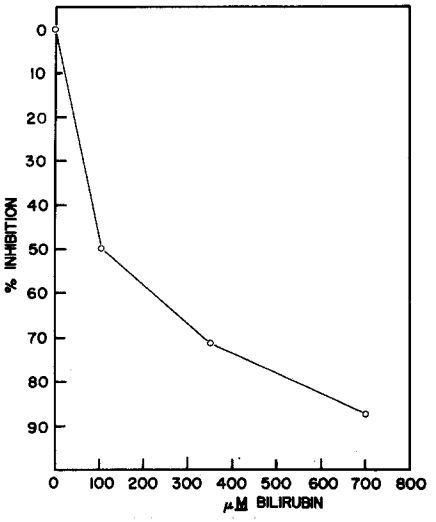

Figure 5 The inhibition of protein synthesis by bilirubin in rat brain cerebellar homogenate protein synthesizing system.

of added bilirubin a striking decrease in the rate of protein synthesis was found (Figure 5). Thus 100 µM of added bilirubin caused a 50% inhibition in protein synthesis and 700 µM of added bilirubin results in an 85% inhibition of protein synthesis. These in vitro results may be reflected in the following

in vivo observations on the Gunn rat. For 48 day old non-jaundiced Gunn rats the cerebellar weight averages 223.4 mg. The RNA content is 0.230 mg/cerebellum and the DNA content is 1.275 mg/cerebellum. Jaundiced littermates had a cerebellar weight of 50.9 mg, an RNA content of 0.053 mg/cerebellum and a DNA content of 0.233 mg/cerebellum. The values of weight, RNA and DNA for the jaundiced animals are comparable to the development of eight day old non jaundiced littermates. These animals have a cerebellar weight of 44.9 mg, RNA of 0.094 mg/cerebellum and DNA of 0.271 mg/cerebellum. The ratio of RNA to DNA in jaundiced and non jaundiced animals is the same, indicating that the composition of cytoplasmic components and nuclear components remains unchanged. Thus the reduction in RNA and DNA in jaundiced animals seems to be a reflection of fewer cells in the cerebellum of these animals.

The present findings are viewed as providing a mechanism for retention of bilirubin in the brain as well as indicating two new pathways by which bilirubin may exert its neurotoxic action. The first of these pathways is the binding of bilirubin to the same sites normally occupied by acidic lipids on the myelin basic protein. The second of these pathways involves the inhibition of cerebellar protein synthesis by a mechanism that is as yet undefined.

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