12. Studies on Kernicterus. III

Developmental Change in Brain Bilirubin Level in Gunn Rats

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Introduction. Bilirubin toxicity has been reported to be due to
the primary interference with mitochondrial functions. However,
these studies have been limited to in vitro situations. It is not clear
that the bilirubin levels used reflected the actual level in the kernicteric
brain since no attempt has yet been made to determine brain bilirubin
content.

In the previous communications, it was reported that the
homozygous Gunn rats show marked cerebellar hypoplasia due to
hyperbilirubinemia, and cerebellar DNA synthesis was impaired in
these mutant rats from 10 days of age. One possible explanation for
such a development-dependency of impaired DNA synthesis might be
that the bilirubin accumulated in the cerebellum from a certain stage
of the development. Special interest will therefore be given to the
investigation of the brain bilirubin level in developing Gunn rat.

In the present communication, bilirubin level in Gunn rat brain
was determined by the method based on chloroform extraction and
direct spectrophotometrical estimation of bilirubin in tissue.

Materials and methods. Male heterozygous (Jj) and homozygous
(jj) Gunn rat littermates born from Jj mother were used in this study.
At least 6 animals were sacrificed in each experiment.

Determination of tissue bilirubin. Animals were anesthetized
with ether and perfused through the left ventricle of the heart with
0.25 M sucrose solution. The brain and liver were removed and homo-
genized by Potter-Elvehjem homogenizer with Teflon pestle with 3
volumes of 0.25 M sucrose solution. The extraction mixture contain-
ing 1 ml tissue homogenate, 4 ml distilled water, 5 ml chloroform, and
12 ml methanol was homogenized. After adding 8 ml of distilled
water, the whole mixture was transferred to a plastic tube, and cen-
trifuged for 30 min at 10,000 × g. The two clear phases and the thin
disc-shaped interphase were then separated. Unconjugated bilirubin
was identified in the lower chloroform phase. These procedures were

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Fig. 1. Postnatal change in bilirubin content (a) and level (b, c) of Gunn rat brain and liver. Bilirubin level is expressed as bilirubin per g wet weight (b) or mg protein (c). At 1 day and 2 days of age, two animals were combined for determining tissue bilirubin. Each point represents mean ± S.D. of 6 samples. ○: heterozygotes; ●: homozygotes.
carried out in dim light at below 4°C. Bilirubin in the chloroform phase was determined from absorption difference at 452 nm and 490 nm on a Hitachi two wavelength double beam spectrophotometer, model 356, using commercial bilirubin as a standard. The recovery of bilirubin in this procedure was approximately 65%. Protein was determined by Biuret reaction with bovine serum albumin as a standard. The regional distribution of bilirubin in Gunn rat brain was determined by a modified procedure using micro-cell. For a determination, six samples were combined.

Results and discussion. Fig. 1a) shows the postnatal change in bilirubin content in Gunn rat brain and liver determined by the method described under “Materials and methods.” The procedure for bilirubin extraction from tissue was based partly on the method of Bratlid & Winsnes6 which was designed for serum bilirubin determination. The liver was taken for a comparative purpose since it also shows highly active DNA synthesis after birth like cerebellum but exhibits neither pathological change7,8 nor impaired DNA synthesis5 in j j Gunn rat. The bilirubin found in j j brain was extremely low, increased gradually after birth until 16 days of age and then decreased. Variation was from 1 to 3 μg. In contrast, the bilirubin content in j j liver increased exponentially after birth. The low amount of bilirubin in j j brain may suggest that too much bilirubin has been used in in vitro studies in which the primary toxicity of bilirubin to mitochondria has been reported.1-3 Diamond and Schmid9 have described that 10.6 μg of bilirubin was transferred per gram of brain in the neurotoxic newborn guinea pig perfused with 14C-bilirubin, and showed that such a bilirubin content could not uncouple oxidative phosphorylation of brain mitochondria. Also, Menken and Weinbach10 have reported the failure to demonstrate impaired oxidative phosphorylation in mitochondria from j j Gunn rat brain. In addition, Schutta et al.11 cast serious doubt

Table I. Regional distribution of bilirubin in Gunn rat brain

| Region              | 8 (ng/protein mg) | 16 (ng/protein mg) | 30 (ng/protein mg) |
|---------------------|-------------------|--------------------|--------------------|
| Cerebellum          | 28.8±1.4          | 24.8±1.2           | 10.6±0.2           |
| Cerebral Cortex     | 27.6±1.5          | 23.4±1.1           | 9.8±0.9            |
| Colliculi           | 27.2±2.0          | 23.7±1.5           | 10.8±1.0           |
| Caudate Nucleus     | 28.0±1.4          | 22.3±0.6           | 9.9±0.8            |
| Thalamus            | 26.8±0.1          | 23.4±0.8           | 9.6±0.8            |
| Hypothalamus        | 27.5±1.7          | 23.0±1.5           | 9.2±0.6            |
| Hippocampus         | 27.4±1.4          | 22.8±1.3           | 9.2±0.6            |
| Brain Stem          | 26.5±1.2          | 24.2±1.1           | 9.9±0.3            |

Each value represents mean ± S.D. of 4 experiments.
from histological observation of jj rat cerebellum upon whether primary bilirubin toxicity was to the mitochondrial functions. In conclusion, it seems premature to discuss primary bilirubin cytotoxicity.

A clear difference was observed in developmental change of bilirubin level between brain and liver (Fig. 1b). Brain bilirubin level decreased after birth in contrast with that in liver which increased steadily and reached a constant level. These findings appear to imply blood brain barrier phenomenon to bilirubin. The tissue bilirubin level expressed in bilirubin per protein content may also be useful in the study of the relationship between an enzyme and bilirubin level in brain. In this expression, the brain bilirubin level in jj rat showed more rapid decrease after birth (Fig. 1c). In addition, as shown in Table I, no selectivity was found in the regional distribution of bilirubin in jj rat brain throughout development.

These observations seem to indicate that the development-dependency of impaired DNA synthesis in jj cerebellum might be due to the unique nature of rat cerebellar development, postnatal neurogenesis, and not to the change in the brain bilirubin level.

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