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Possible Mechanism of Reduced Thymidine Kinase Activity in Gunn Rat Cerebellum

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Introduction. In the previous communication, it has been reported that the activity of cerebellar thymidine kinase in homozygous (jj) Gunn rat with hereditary hyperbilirubinemia was reduced considerably as compared with that in heterozygous (Jj) littermate from very early stage of development, 6 days after birth. This decrease in thymidine kinase activity preceded by at least 4 days the decrease in wet weight, DNA content and 3H-thymidine incorporation into DNA in jj cerebellum.

A major question that emerges from these results is whether such a decrease is due to the inhibition of enzyme by bilirubin, or to the impaired induction of thymidine kinase.

In the present communication, the possibility that bilirubin might inhibit cerebellar thymidine kinase was investigated in vitro, and the results were evaluated in reference to their significance in vivo.

Materials and methods. Thymidine kinase activity was assayed by the method of Taylor et al. as previously described. The cerebella of 12 day-old Jj rats were used for enzyme preparation. Bilirubin solution was prepared immediately before use by dissolution in 0.1 M Na₂CO₃ and dilution with 0.1 M Tris-HCl buffer, pH 7.5. In the experiment of bilirubin inhibition, 10 µl of bilirubin solution was added to reaction mixture bringing the final volume to 200 µl.

Bilirubin levels of each subcellular fraction of brain and liver in jj Gunn rat were determined as previously reported. Twelve day-old jj rats were anesthetized with ether and perfused with 0.25 M sucrose. The removed brain and liver were homogenized with 0.25 M sucrose, centrifuged at 105,000 g for 60 min. Bilirubin contents of homogenate, 105,000 g supernatant, and pellet suspended in 0.25 M sucrose were determined.

Results and discussion. As shown in Table I, bilirubin inhibited cerebellar thymidine kinase activity in vitro. Approximately 15%
inhibition of thymidine kinase activity was observed at bilirubin concentration of $10^{-5}$ M, and 30% at $10^{-4}$ M. The mode of inhibition by bilirubin was found to be competitive from Lineweaver-Burk plots (Fig. 1). The apparent $K_m$ of cerebellar thymidine kinase was 2.5 $\mu$M.

In order to evaluate the results of such in vitro inhibition, bilirubin levels in vivo and in the reaction mixture used above were compared (Table II). To determine cerebellar bilirubin levels in jj rats, whole brains of 12 day-old jj rats were used because no difference was found in bilirubin levels between cerebellum and other regions of jj brain.\(^5\) The jj liver was also used for comparison. In brain, only about 10% of bilirubin in homogenate was found in the 105,000 g supernatant fraction, i.e., the crude enzyme fraction in the assay of thymidine kinase. The bilirubin level of brain supernatant was about 7 ng/mg protein, while, the level in the reaction mixture was about 27 ng/mg protein.

Table I. Effect of bilirubin on cerebellar thymidine kinase activity

| Bilirubin concentration ($\times 10^{-5}$M) | Enzyme activity (cpm/mg protein $\times 10^{-4}$) | Inhibition (%) |
|--------------------------------------------|-----------------------------------------------|---------------|
| 0.0                                        | 17.9                                          | 0.0           |
| 0.5                                        | 15.7                                          | 12.6          |
| 1.0                                        | 15.4                                          | 14.0          |
| 5.0                                        | 14.3                                          | 20.1          |
| 10.0                                       | 13.1                                          | 27.0          |

The assay was performed as described previously\(^1\) using cerebella of 12 day-old heterozygous Gunn rats as enzyme source.

Fig. 1. Effect of bilirubin on cerebellar thymidine kinase activity; double reciprocal plots of enzyme activity vs thymidine concentration. The assay system was as described previously.\(^1\) The assay was performed in the presence of $10^{-4}$M bilirubin using cerebella of 7 day-old heterozygous Gunn rats as enzyme source.
mixture in inhibition study was 10,000 ng/mg protein at bilirubin concentration of 10^{-5} \text{M}, indicating a level as high as 1,000 times the actual in vivo level. Thus, it seems unlikely that inhibition of thymidine kinase by bilirubin occurs in j j Gunn rat cerebellum.

In addition, about 50\% of bilirubin was recovered in supernatant fraction in liver. This excessive distribution of bilirubin seems to be the reflection of bilirubin binding with ligandin which exists in supernatant fraction of liver.6 It has been known that no abnormality was detectable in liver in hyperbilirubinemia,1,3,7 though it also shows highly active DNA synthesis after birth and high bilirubin level in j j Gunn rat.5 It appears possible that ligandin-binding may prevent bilirubin cytotoxicity in liver. However, it should be noted that no correlation was observed between the developmental change in brain bilirubin level5 and cerebellar thymidine kinase activity in jj animals.1 Furthermore, bilirubin did not selectively accumulate in cerebellum in jj brain.5

These findings seem to imply that the synthesis of thymidine kinase may be decreased in jj cerebellum. Recently, the possibility of impaired brain protein synthesis by bilirubin has been reported.8,9 It has been demonstrated that protein synthesis is essential for DNA replication in mammalian cells.10,11 However, it is not clear at the present time whether the impaired induction of cerebellar thymidine kinase in jj Gunn rat is due to the direct affect of bilirubin on cere-

|                | Brain Bilirubin content | Brain Bilirubin level | Liver Bilirubin content | Liver Bilirubin level |
|----------------|-------------------------|-----------------------|-------------------------|-----------------------|
| homogenate     | \mu g \% (100)          | 18.0 (100)            | 13.0 (100)              | 175                   |
| 105,000 \times g supernatant | 0.069 (10.6) | 6.9 (44.3) | 5.8 (175) |
| precipitate    | 0.611 (94.3)           | 25.4 (106.1)          | 8.1 (175)               | 222                   |
| recovery       | (104.9)                | (106.1)               |                         |                       |

The brains and livers of 12 day-old homozygous Gunn rats perfused with 0.25M sucrose were homogenized with 5 volumes of 0.25M sucrose, centrifuged at 105,000 \text{g} for 60 min. Bilirubin in homogenates, supernatants, and pellets were extracted by chloroform and determined photometrically as previously reported.9 Bilirubin level was expressed as ng bilirubin per mg protein.
bellar proliferative cells, or to the interference with an unknown regulatory mechanism of Purkinje cell in cerebellar cell proliferation.

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