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# BILIVERDIN INITIATES THE LIVER REGENERATION IN THE RAT — A HYPOTHESIS

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SUMMARY: Biliverdin has been observed to occur in the blood plasma of a 90% hepatectomized rat. It is also shown that the bile pigment induces a rise in mitotic index in the hepatic parenchymal cells of an intact rat at about 30 h and an elevated rate of hepatic DNA synthesis at 26 h after a single intraperitoneal injection. Hemoglobin, bilirubin, hemin, and protoporphyrin exhibited some of the inducing activity at lower degrees. It is hypothesized that biliverdin initiates the liver-cell-multiplication.

A normal human liver is capable of conjugating and excreting three times as much bilirubin as the amount present in the daily physiologic load and still can maintain within normal range the concentration of it in the blood (1). On the other hand it is said that biliverdin, the precursor of bilirubin, does not occur in normal sera (2) but it can be detected in the serum of patients with obstruction of common bile duct, liver cirrhosis, catarrhal jaundice, etc. (3-5). This may mean that a normal liver does its best to eliminate biliverdin from the serum but once the liver fails to remain normal, some amount of biliverdin starts appearing in the blood as a result of the reduced capacity of the organ except in the case of obstruction of common bile duct, in which the cause of appearance of biliverdin in the serum is a re-oxidation of bilirubin. Taking these relationships into consideration and specu-

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lating on the mechanism of onset of liver regeneration after partial hepatectomy, which has been a subject of much research (e.g. 6-8), the first author has reached a hypothesis that the pigment biliverdin might be the initiating factor for the regeneration.

Among circumstances supporting the above hypothesis are the following:

1. It has been demonstrated that the initiating factor appears to be humoral (e.g. 8-11).

2. Senescent red blood cells are destroyed and hemoglobin is released at a constant rate. The life spans of red blood cells of man, dog, and rat range 108-135, 90-135, and 50-60 days, respectively (12).

3. Since biliverdin is formed from hemoglobin, the rate of biliverdin production should also be constant except for diseases.

4. It is likely that Nature has chosen as the controller of liver size a compound that gives the organ a load that is quantitatively constant and requires the organ's full and consistent activity.

The purpose of this communication is to demonstrate the brief presence of biliverdin in the rat blood plasma after a 90% hepatectomy and the rises in the mitotic index and in the rate of DNA synthesis in liver cells of rats that have been intact except for the intraperitoneal injections of the pigments mentioned above as well as hemin and protoporphyrin.

## MATERIALS AND METHODS

Female Wistar rats were obtained either from Nakajima Experimental Animal Institute (Kasugai, Aichi, Japan) or from Chubu Kagaku Shizai Company (Nagoya, Japan), freely given food and water at all times, and were used when they weighed 300-400 g in the plasma biliverdin experiment and when they were about 150 g in the mitotic index and DNA synthesis studies. Biliverdin, bilirubin, hemoglobin (bovine), and hemin were purchased from Sigma Chemical Company, protoporphyrin from Nakarai

Chemicals (Kyoto, Japan), globin from Tokyo Kasei Industries (Tokyo), and [Me-$^3$H]thymidine from New England Nuclear.

The plasma biliverdin was estimated by reading absorbance at 660 nm (13) as follows:

The rat was decapitated at a scheduled time either after a sham operation (opening and closing the hypochondrial area with no liver excision) or after a 90% hepatectomy, in which only the accessory lobe had been left intact (8), and blood was collected in a test tube containing 0.05 ml of heparin solution, 5,000 I.U. per ml. The tube was centrifuged at 600 x g and 4° for 10 min and 0.8 ml of the supernatant was mixed with 2 ml of chilled methanol in a stoppered tube. The tube was kept ice-cold for 10 min, centrifuged at 5,000 x g and 4° for 10 min and the supernatant combined with 1 ml each of benzene and $CCl_4$ and 0.05 ml of 1 M NaOH in another stoppered tube which was then vigorously shaken 100 times at 4°. The optical density of the final supernatant at 660 nm was measured in Hitachi Spectrophotometer and the absorbance was compared with the reference which had been obtained with known amounts of biliverdin added to normal rat plasma. The reference absorbances for 10 and 20 μg of the pigment in each milliliter of plasma were 0.035 and 0.070, respectively.

The injected compounds were dissolved in 0.14 M NaCl containing 0.1 M NaOH. Injections were made intraperitoneally under a light ether anesthesia except for those of [$^3$H]thymidine which were made in the jugular vein. The control rats received 1 ml per 100 g body weight of the above solvent.

For evaluations of the mitotic indexes the rats were killed by cervical dislocation and the right lateral lobe of the liver was fixed in Carnoy's solution, 99.8% ethanol, and absolute ethanol and embedded in paraffin. Sections of 5 μm in thickness were stained with hematoxylin and eosin. Mitotic indexes were measured according to Brues and Marble

(14) except that the parenchymal cells that were seen in 120 fields under a light microscope at the magnification of 200 were checked. The average number of parenchymal cells seen in each of these fields counted with 12 of them was 1,185 ± 30 (mean ± S.E.) which was used throughout the mitosis study.

DNA synthesis in vivo was estimated by the incorporation of [Me-$^3$H]-thymidine into nuclear DNA. Ten μCi of [Me-$^3$H]thymidine were injected in the right jugular vein of the rat fixed on a metal board, which had been devised by Dr. Asaharu Kanetake, Laboratory of Biological Products, Institute of Medical Science, University of Tokyo, at either 26 or 26.5 h after the single intraperitoneal injections of the compounds. After 2 h of the pulse period the animals were killed and the liver nuclei were isolated with the citric acid method according to Lieberman et al. (15). The nuclei were washed with about 10 ml of 0.3 M trichloroacetic acid on a filter covered with approximately 200 mg of cellulose powder at 4°. The filter cake was dried up, dispersed in 0.5 ml of 0.68 M perchloric acid, incubated at 70° for 20 min, and counted in a phosphor solution. The counting efficiency was approximately 37%. Concentration of DNA in the phosphor solution was measured with the diphenylamine method (16).

## RESULTS AND DISCUSSION

The amount of biliverdin in the blood plasma of intact or sham operated rats was practically nil, i.e. the final supernatant prepared as described in the previous page had absolutely no absorbance against water nor against methanol-water mixture treated in the same way as for the above supernatants.

Those values for 90% hepatectomized rats killed at 5, 10, 20, 30, 45, 60, 120, and 180 min after the time of ligation of the blood vessels and bile ducts serving the right lateral and caudate lobes (all of these vessels were ligated in a bundle and the left lateral and median lobes

had been removed immediately beforehand) were 3.2, 2, 1.3, 1.3, 2, 1.7, 0.7, and 0.7 μg per ml plasma, respectively. Another series of experiments gave the comparable values of 4.2, 0.7, 0.7, 1.3, 1, 1.7, 0.7, and 0.3, respectively. They vary a great deal presumably because of various conditions under which the rats were put during the operation, individual differences between the animals, or both, however, it seems safe to say that certain amounts of biliverdin do exist in the blood plasma of 90% hepatectomized rats at least during the period between 5 and 180 min after the operation. Those values for 70% hepatectomized rats (17) were even more inconsistent ranging from zero to 2.3 μg per ml plasma. It may be useful to add that none of the supernatants for measurement of absorbance was turbid and that those with some absorbance were slightly but definitely greenish.

As for the mitosis study most rats were killed at 30 h after injection because maximal mitotic index was observed at this time as tested with 10 mg per 100 g body weight of biliverdin as shown in Table I.

The reason why the maximal mitotic index is observed at 30 h instead of 24-28 h as in the case of 70% hepatectomy (7, 14, 17-21) is not known but it may be because of the absence of the increased portal blood pressure which accompanies the 70% hepatectomy (22) and may accelerate the distribution of the initiating factor for the regeneration to the remaining liver cells.

The effects of other compounds that were more or less related to biliverdin were also examined besides that of biliverdin at various doses and most of the results are shown in Table II.

Not shown in Table II are the results with 1 ml per 100 g body weight (the last five words will be omitted hereafter) of 0.14 M NaCl containing 0.1 M NaOH (the control), 15 and 20 mg of biliverdin, 40 mg of hemoglobin, 10 mg each of hemin and protoporphyrin, and 4 mg of globin, which were 3.7 ± 2.1 (2), 507 (1), 484 (1), 411 (1), 176 ± 20

TABLE I

Mitotic Indexes per 100,000 Hepatic Parenchymal Cells at Various Times after Single Intraperitoneal Injections of 10 mg per 100 g Body Weight of Biliverdin

| 28 h | 30 h | 32 h |
|---|---|---|
| 466 ± 118 (2) | 862 ± 68 (5) | 224 ± 8 (2) |

Means ± S.E. (number of animals) are given.

(2), 73 ± 33 (2), and 3.3 ± 0.4 (2), respectively. Among the compounds tested, i.e. biliverdin, bilirubin, hemoglobin, hemin, and protoporphyrin, biliverdin was the most potent in forcing the normal liver cells to divide, hemoglobin being the second most potent. In order to check the possibility that the globin moiety in hemoglobin should be responsible for the potency, the effect of globin was tested and found negative.

The question of whether these active compounds besides biliverdin are converted to biliverdin before displaying their activities of inducing the liver-cell-multiplication or they originally possess the activity has not been investigated, however, it may be of interest to note that all of these compounds can be converted to biliverdin while biliverdin cannot change into any of these except bilirubin.

No sign of cell damage was detected in any of the cells observed under a light microscope and few figures of mitosis were found among the hepatic cells besides parenchymal cells. Mitotic index was also checked with a remnant liver at 28 h after a 70% hepatectomy, which turned out to be 397 per 100,000 parenchymal cells.

As another line of evidence for biliverdin as well as for hemoglobin to induce an elevated frequency of hepatic cell-division, the effects of

TABLE II

Mitotic Indexes per 100,000 Hepatic Parenchymal Cells at 30 h after Single Intraperitoneal Injections of Various Compounds at Various Doses

| Dose | Biliverdin | Bilirubin | Hemoglobin |
|---|---|---|---|
| 0.1 | 130 ± 47 (2) | 129 ± 2 (2) | 26 ± 2 (2) |
| 0.4 | 44 ± 4 (2) | | 14 ± 8 (2) |
| 1 | 92 ± 5 (2) | 106 ± 1 (2) | 87 ± 5 (2) |
| 4 | 194 ± 108 (2) | | 603 ± 148 (2) |
| 10 | 862 ± 68 (5) | 20 ± 8 (6) | 681 ± 134 (2) |

Doses are expressed as mg equivalent biliverdin per 100 g body weight. The doses of hemoglobin were calculated with the molecular weight of 68,000 and the number of heme moieties per mole of 4. Means ± S.E. (number of animals) are given.

these compounds on the rate of synthesis of hepatic nuclear DNA were examined as already described. Results are expressed as dpm per mg DNA. The results with the rats who received 10 mg per 100 g body weight of biliverdin, were pulsed at 26 h, and were killed 2 h later were 7250 ± 550 (2) dpm per mg DNA (mean ± S.E. (number of animals)). Comparable data of 10 mg biliverdin and the pulse time of 26.5 h were 2440 ± 60 (2). Those for 4 mg equivalent biliverdin of hemoglobin and the pulse times of 26 and 26.5 h were 3620 ± 1580 (2) and 2820 ± 170 (2), respectively. The control rat receiving 1 ml per 100 g body weight of 0.14 M NaCl containing 0.1 M NaOH and the pulse at 26.5 h gave 600 dpm per mg DNA. These data are in accord with those of the mitotic index.

MacDonald and Pechet (23) have reported that rat liver cells show a marked increase in DNA synthesis and in mitosis following ligation of the common bile duct beginning 2 days after obstruction and continuing as long as obstruction is present. They concluded as follows: The

cause of the marked liver cell proliferation was not determined although it was theorized to be due to the effects of bile. It may be said that their theory has been approved by the present study. However, the idea that biliverdin initiates the liver-cell-multiplication remains hypothetic because the possibility of intervention of some mediator(s) has not been excluded.

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