Transfer of Bilirubin Through the Liver of Gunn Rats

It is believed that conjugation of bilirubin is an obligatory step for its excretion in the bile[1,2]. In contradiction to this current belief, OSTROW[3] suggested that unconjugated bilirubin (UB) may be secreted as such by the liver cell. BERTHELOT and FAUVERT expressed[4] the same view, but did not consider this excretion as physiologically important. OKOLICSÁNYI et al.[5] supposed that some conjugated bilirubin (CB) might be hydrolyzed by the liver cells of Gunn rats and then excreted in the bile. BILLING[6] observed recently that relatively large quantities of UB are found in the bile of Gunn rats after i.v. injection of large doses of this pigment.

It seems therefore that UB may be excreted in the bile. We have investigated this poorly understood phenomenon in Gunn rats because of their congenital incapacity to form CB[7].

1. In order to exclude the possibility of an active transfer of UB from the plasma to the bile, we infused increasing doses of this pigment intravenously. At different times after the beginning of infusion, plasma and bile were analyzed for total bilirubin (TB).

2. In order to define how this passage occurs, through the hepatocytes or between them, we infused i.v. large concentrations of UB in association with Novobiocin. Again, plasma and bile samples were analyzed for TB at different times after the beginning of the infusion.

Material and methods. 22 homozygous Gunn rats were used for these experiments. The animals were anaesthetized with sodium pentobarbitone (30 mg/kg i.p.), a cannula was placed into the trachea and the rats were kept under the heat of a common lamp. The common bile duct and the 2 femoral veins were then cannulated with polyethylene tubing to collect bile and blood and to administer UB.

Experiment A: A primary dose of 2 mg/kg of UB (British Drug Houses) was injected i.v. to 16 Gunn rats. Immediately thereafter, 5 of them received i.v. 15 mg, 6 of them 30 mg and the remaining 5, 45 mg of UB. This was freshly dissolved in 5 ml of 0.1 *M* sodium carbonate and infused at a constant rate of 0.2 ml/min. Bile and blood samples were collected before and 5, 10, 15, 20 min after the injection of UB. The samples were immediately stored at 0–4 °C. The blood was centrifuged for 10 min at 0–4 °C to separate the plasma. Bile and plasma samples were analyzed for TB by a modification[8] of the MICHAELSSON's microassay[9].

Experiment B: 6 Gunn rats received i.v. 2 mg of UB dissolved in 0.1 *M* sodium carbonate. Then 4 mg of Novobiocin (Albamycin, Upjohn Co.) per 100 g body weight dissolved in 1 ml of 0.9% NaCl, and 30 mg of UB freshly dissolved in 4 ml of 0.1 *M* sodium carbonate, were combined and given i.v. by means of an infusion pump at a constant rate of 0.2 ml/min. Blood and bile samples were collected and analyzed as described above.

Results and discussion. Figure 1 represents data obtained after the i.v. infusion of increasing amounts of UB. The total bile bilirubin concentration increased 5 min after the beginning of the infusion in each series of experiments and remained high over the whole period of observation. Plasma and bile bilirubin concentrations were not significantly different.

However, when 45 mg of UB were administered, plasma and bile bilirubin concentrations were different, 15 min and 20 min after the beginning of infusion; we interpret this phenomenon as due to the toxic action of UB, as these animals developped dyspnea and progressive decrease of bile flow shortly after the beginning of infusion. On the contrary, this was not the case when lower (30 mg, 15 mg) UB doses were infused.

Our data do not prove an active transfer of this pigment. Previous studies of WEINBREN and BILLING[10] have shown that a dose of 20 mg of UB for a normal rat of 250 g is sufficient to saturate the excretory capacity of the liver for bilirubin. In our experiments it was not possible to saturate the excretory capacity of Gunn rat liver for bilirubin. However, it cannot be excluded that we have partly measured diazo-positive metabolites of bilirubin[7].

BILLING et al.[11] demonstrated that Novobiocin interfers with the conjugation, uptake and excretion of bilirubin. The same authors, in in vitro experiments with plasma

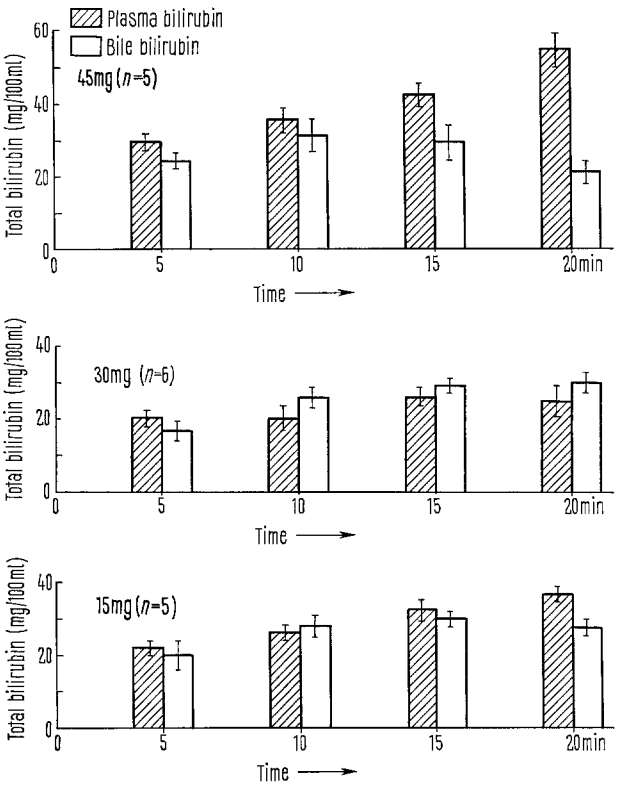

Fig. 1. Gunn rats. Infusion of unconjugated bilirubin. Mean changes of total plasma and bile bilirubin concentrations after i.v. infusion of increasing amounts of unconjugated bilirubin. Vertical bars indicate standard errors.

[1] I. M. ARIAS, L. JOHNSON and S. WOLFSON, Am. J. Physiol. *200*, 1091 (1961).
[2] R. SCHMID, J. AXELROD, L. HAMMAKER and R. L. SWARM, J. clin. Invest. *37*, 1123 (1958).
[3] J. D. OSTROW, J. clin. Invest. *46*, 2053 (1967).
[4] P. BERTHELOT and R. FAUVERT, Rev. fr. Etud. clin. biol. *12*, 702 (1967).
[5] L. OKOLICSANYI, P. MAGNENAT and J. FREI, Lancet *1*, 1173 (1968).
[6] B. H. BILLING, Personal communication (1968).
[7] R. SCHMID and L. HAMMAKER, J. clin. Invest. *42*, 1720 (1963).
[8] L. OKOLICSÁNYI, J. FREI and P. MAGNENAT, Enzym. biol. clin., in press (1970).
[9] M. MICHAELSSON, Scand. J. clin. Invest. *13*, 1 (1961).
[10] K. WEINBREN and B. H. BILLING, Br. J. exp. Path. *37*, 199 (1956).
[11] B. H. BILLING, Q. MAGGIORE and G. GOULIS, *The Biliary System* (Blackwell Sci. Publ., Oxford 1965), p. 215.

from rats which had received infusions of both UB and Novobiocin, did not find any interaction between the 2 substances. Therefore, we used Novobiocin to confirm our hypothesis that the passage of UB occurs from the liver cell to the bile.

In our experiments, the total plasma bilirubin concentration was higher in Novobiocin treated rats than in the control group, and the concentration of TB in the bile was lower. Statistical analysis of variance showed that these differences were significant (plasma: $F = 13.8$, $p < 0.01$; bile: $F = 6.7$, $p < 0.02$). If the passage of bilirubin to the bile did not occur through the liver cell, plasma and bile bilirubin concentrations would not be affected by Novobiocin.

Intravenous infusion of CB permits biliary and urinary excretion of UB[12,13]. Our experiments suggest that under certain conditions UB may be excreted in the bile even in the absence of CB[14].

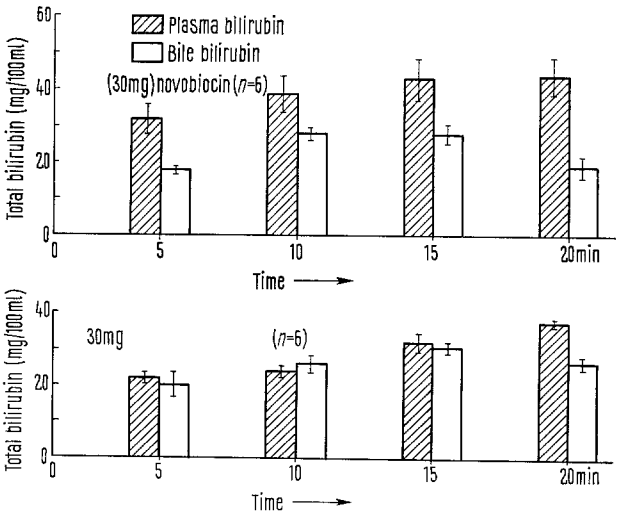

Fig. 2. Gunn rats. Infusion of 30 mg of unconjugated bilirubin. Mean changes of total plasma and total bile bilirubin after i.v. infusion of unconjugated bilirubin (below) and unconjugated bilirubin + Novobiocin (above). Vertical bars indicate standard errors.

Résumé. L'absence d'un T_m lors de perfusion en quantité croissante de bilirubine non conjuguée chez le rat Gunn, ainsi que la diminution de la concentration biliaire de la bilirubine totale lors d'infusion simultanée de bilirubine non conjuguée et de Novobiocine, suggèrent la possibilité d'un transfert passif de la bilirubine non conjuguée du sang dans la bile, à travers la cellule hépatique.

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[12] E. W. CALLAHAN JR. and R. SCHMID, Gastroenterology *57*, 134 (1969).

[13] E. A. RODRIGUEZ GARAY, M. DEL ROSARIO SPETALE, B. NOIR and G. SIVORI, Experientia *25*, 494 (1969).

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Genetic Variation of Supernatant and Mitochondrial Malate Dehydrogenase Isozymes in the Teleost *Fundulus heteroclitus*

The study of isozymes has provided considerable insight into the subunit structure of enzymes[1] as well as providing gene markers for the analysis of problems in development[2] and evolution[3]. The isozyme system of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) is represented by 2 major forms in vertebrates and invertebrates: 1) supernatant malate dehydrogenase (S-MDH), and 2) mitochondrial malate dehydrogenase (M-MDH). In addition to their different subcellular locale these MDHs differ in their electrophoretic mobility[2], kinetic behavior[4], amino acid composition[5], and antigenic properties[6]. Both the S-MDH and M-MDH of vertebrates have been shown to exist as dimers by in vitro molecular hybridization[7] and allelic isozyme variation[8–10]. Malate dehydrogenases exist as conformational isozymes in some organisms[1,11].

The malate dehydrogenase isozymes of *Fundulus heteroclitus* were investigated because this species is potentially valuable for studying developmental and biochemical genetics. The present paper describes the malate dehydrogenase isozymes of *F. heteroclitus*, their subcellular distribution and discusses their possible genetic and molecular bases.

Methods. Sexually mature *F. heteroclitus* of both sexes were trapped from Mill Pond in Woods Hole, Massachusetts. The subcellular distribution of MDH isozymes from freshly killed *F. heteroclitus* was determined by differential centrifugation. Fresh unfrozen livers were homogenized in a loose-fitting Dounce homogenizer, in 2 volumes of 0.25 *M* sucrose in 0.1 *M* *Tris*-HCl, pH 7.7. Centrifugation was carried out in an SS-34 rotor in a Sorvall RC 2-B at 4 °C. Unbroken cells, cell fragments, and nuclei were removed by 480 *g* for 5 min. The mitochondria were precipitated by 12,100 *g* centrifugation for 10 min. The supernatant resulting from centrifugation at 105,000 *g* for 40 min contains the supernatant MDH (S-MDH). The isolated mitochondria were twice washed and subjected to 12,100 *g* for 10 min and then Dounce-homogenized in one volume of 0.1 *M* *Tris*-HCl, pH 7.0 followed by centrifugation at 105,000 *g* for 30 min. This supernatant contains the malate dehydrogenase released from the mitochondria (M-MDH).

The skeletal muscle from each of 245 frozen *Fundulus* was homogenized in a Dounce homogenizer in one volume of 0.1 *M* *Tris*-HCl, pH 7.0, centrifuged at 48,000 *g* for 30 min at 4 °C prior to the electrophoreses used for the population analysis.

All electrophoreses were performed in a 14% vertical starch gel at pH 6.9. The stock buffer was 0.75 *M* *Tris* + 0.25 *M* citric acid (monohydrate) adjusted to pH 6.9.