Intrahepatic Distribution of Tritiated Bilirubin in Normal and Gunn Rats: Subcellular Fractionation.* (32338)

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(Introduced by E. H. Wood)
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The mechanisms involved in hepatic uptake, storage, conjugation, and excretion of bilirubin are incompletely understood. Brown and coworkers(1) used bilirubin-^{3}H to establish the localization of the pigment within the normal rat liver cell. They found the highest concentration of radioactivity in the supernatant fraction. Our study was undertaken to compare the uptake and distribution of unconjugated bilirubin-^{3}H in normal and in Gunn (glucuronyl-transferase deficient) rats. While this study was in progress, Bernstein and coworkers(2) described the intracellular distribution of unconjugated and conjugated bilirubin-^{3}H in normal and in Gunn rats.

Materials and methods. Preparation of purified tritiated bilirubin. Commercially prepared bilirubin-^{3}H, by the standard Wilzbach technique, was obtained from Tracerlab (Waltham, Mass.); 1 mg of this material (specific activity, 0.7 mc/mg) was dissolved in freshly prepared 0.1 M sodium bicarbonate, the pH was adjusted to 7.8 with 0.1 N HCl, and an equal volume of rat serum was added. This mixture was injected into 300-g male Sprague-Dawley rats. Prior to the injection, polyethylene catheters were placed in the tail vein and common bile duct of the rats (ether anesthesia). Bile was collected in an ice bath in darkness for 4 hours. Bilirubin was crystallized from the bile(3) and recrystallized until it reached a constant specific activity. For quantitation, crystals were dissolved in chloroform and the bilirubin concentration was measured by direct spectrophotometry at 450 mμ, with a standard curve prepared with commercial bilirubin (Mann Research Laboratories, Inc., New York).

An aliquot of the chloroform solution containing 3 to 6 μg of bilirubin was pipetted into counting vials, dried at room temperature, and redissolved in 1 ml of phenethylamine (Matheson, Coleman & Bell). To each sample was added 20 ml of Liquiflor (T.M. Pilot Chemical, Inc., Watertown, Mass.) diluted with toluene. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 314 Ex and Model 4322), with toluene-^{3}H as the internal standard. Counting efficiency ranged from 8 to 9% with Model 314 Ex and 15 to 25% with Model 4322.

After the fifth recrystallization, the specific activity was 0.55 to 0.59 μc/μg. About 5 to 10% of the original unpurified bilirubin was recovered as purified bilirubin with a constant specific activity.

Procedure. Five male Gunn rats and 4 normal rats were anesthetized with ether, and their bile ducts were cannulated. Into each was injected 30 to 50 μg of tritiated bilirubin-^{3}H as previously described(1). Each rat was killed 5 minutes after the injection. Radioactivity was determined in aliquots of serum and bile by using Kinard Scintillator fluid. The livers were rapidly excised, chilled to 0 C, and weighed. A small portion was placed in Dalton's solution(2) for 1 hour and then rapidly dehydrated in graded alcohols. Aliquots of the Dalton solution and alcohol were counted to determine loss of radioactivity. Also, 5 g of liver was homogenized in 15 ml of 0.25 M sucrose(4). An aliquot of the homogenate was also counted in Kinard Scintillator fluid. The homogenate was centrifuged for 10 minutes (600 $\times$ g) in a refrigerated centrifuge for separation of the nuclei fraction. The supernatant fraction was poured off and the sediment was washed twice with 5 ml of the sucrose solution. The collected supernatant fractions were centrifuged for separation of the mitochondrial fraction (10 minutes, 8,500 $\times$ g) in a refrigerated

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TABLE I. Distribution of Tritiated Bilirubin in Liver Cells of Normal and Gunn Rats.

Rat	Nuclei (%)	Mitochondria (%)	Lysosome (%)	Microsome (%)	Supernate (%)	Recovery (%)
			Normal			
1	10.3	3.3	2.4	10.4	54.4	80.8
2	11.0	3.0	2.4	5.6	52.1	74.1
3	9.3	13.4		13.9	41.6	78.2
4	8.4	10.8		9.7	44.2	73.1
		(mito. + lyso.)				
Mean	9.7	8.8		9.9	48.1	76.6
± S.D.	±1.2	±3.9		±3.4	±6.1	±3.6
			Gunn			
5	10.0	15.0	7.0	16.0	48.0	96.0
6	3.9	6.4	10.0	6.4	54.6	81.9
7	6.7	7.6	6.2	5.8	72.2	98.5
8	9.4	3.6	3.9	17.8	61.1	95.8
9	9.0	7.1		11.8	48.8	76.7
		(mito. + lyso.)				
Mean	7.8	13.5		11.6	56.9	91.0
± S.D.	±2.6	±6.3		±5.5	±10.0	±10.6

centrifuge; this supernate was taken off with a pipette and the residue was washed twice with sucrose solution. Half of the collected supernate was used for separation of the lysosome fraction. The other half was centrifuged for separation of the microsomal fraction (30 minutes, 100,000 × *g*) and washed twice with sucrose solution. Aliquots of each fraction and of the final supernate were diluted with Kinard Scintillator fluid and counted.

Results. Approximately 85% of the injected isotope was accounted for in the liver, serum, and bile. The distribution of bilirubin-^{3}H among the subcellular components of the hepatic cells in the normal rat and in the Gunn rat is shown in the Table. In all experiments, the greatest amount of radioactivity was recovered from the supernatant fraction.

We found, as did Bernstein and associates (2), a slight increase in the radioactivity in the supernatant fraction of Gunn rats compared with normal rats. However, we did not find a corresponding decrease in activity in the microsomal fraction. In both groups of animals, recovery of label in the lysosomal remained low.

Discussion. The use of bilirubin-^{3}H permits measurement of bilirubin distribution in tissues at physiologic concentrations. The stability of unconjugated bilirubin-^{3}H *in vitro* is supported by the observations by Bernstein and associates (2) of virtually identical hepatic uptake and subcellular distribution of ^{3}H-labeled and ^{14}C-labeled unconjugated bilirubins in normal rats. The pattern of subcellular distribution in this study corresponds closely with that found by other investigators (1,2). Although electron microscopic observations (5,6) have suggested that lysosomes may play a role in bilirubin transport and excretion, we demonstrated that only 10% or less of the radioactivity was present in the lysosomal fraction at a time of maximal intrahepatic distribution. Under physiologic conditions, hepatic uptake and transport of bilirubin is rapid, and it appears unlikely that lysosomes play a major role in this process.

Summary. The hepatic intracellular distribution of unconjugated bilirubin-^{3}H after its intravenous injection into normal and Gunn (glucuronyl-transferase deficient) rats was studied and found to be similar. The major site of radioactivity was the supernatant fraction in all experiments. It is doubtful that lysosomes play a major role in the transport of bile pigment.

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Opsonic Property of Multiple Myeloma Serum.* (32339)

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Bacterial infections are a frequent and often fatal occurrence in patients with multiple myeloma(1). Despite a number of studies of host defense mechanisms, disagreement still exists as to specific defects. Three broad host factors have been considered. First, multiple myeloma may be associated with a suppression of antibody synthesis. Second, there may develop a diminution in the degree of delayed hypersensitivity. Third, the hyperglobulinemia may result in alterations in the phagocytic capabilities of leucocytes. Both Ingram and Penny and Galton utilizing iron granules or heat-inactivated yeast cells as particles have demonstrated inhibition of phagocytosis(2,3). This defect was related to the abnormal amounts of globulin present in the serum.

The following studies were directed toward assessment of opsonic activity as a possible additional serum defect. By employing a bacterial strain which is both ingested and killed by leucocytes in the presence of a heat-labile serum factor, the opsonic activity of serum from patients with multiple myeloma can be measured.

Materials and methods. Seven patients with multiple myeloma were selected from the hematology service at Parkland Memorial Hospital. The definitive diagnosis of multiple myeloma was based on the classical clinical features and morphological criteria of sheets of malignant-appearing plasma cells in the bone marrow.

Blood was drawn from the patients with multiple myeloma and from normal controls, the serum was separated and stored at −5°C and was used within 2 weeks to maintain its natural opsonins. Neither patients nor controls were on antibiotics at the time that the specimens were obtained.

The *in vitro* phagocytosis technique was basically that described by Hirsch and Strauss, substituting human leucocytes and a strain of *E. coli* 0-4 in their test procedure(4). Blood was obtained from normal antepartum patients and leucocytes were separated by a modification of the technique of Rogers(5). Siliconized glassware was used throughout the experiment. Twenty milliliters of blood were added to 10 ml of heparinized 3% dextran in saline. Blood was allowed to settle for 30 minutes at 37°C. The white cell rich supernatant was aspirated with a Pasteur pipette, transferred to a conical centrifuge tube and centrifuged at 1000 rpm for 7 minutes. The supernatant was discarded, and the white cell sediment washed twice in heparinized saline. The cells were then resuspended in Hanks' balanced salt solution containing barbital buffer (0.1 M, pH 8.6) and 0.1% gelatin to a concentration of approximately 2×10^7 cells per ml.

E. coli 0-4 were grown for 18 hours in trypticase soy broth (BBL), centrifuged at 8000 rpm for 10 minutes and washed twice in Hanks' solution. They were then resuspended in Hanks' solution to an optical density of 0.6 at 650 λ in a Bausch and Lomb spectronic

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