Labeled Bilirubin Production by a Clonal Strain of Rat Hepatoma Cells[1] (35340)

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(Introduced by Frederick Stohlman, Jr.)
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Although bilirubin is derived principally from the hemoglobin of senescent erythrocytes, a small, but significant, fraction arises from nonhemoglobin sources (1–3). The liver appears to be the major source of this bilirubin fraction (4–6). However, the type of liver cell in which this fraction is formed has not been identified, and its metabolic origins are poorly understood (7). A promising approach to these problems seemed to lie in the study of a clonal strain (MH_1C_1) of rat hepatoma cells which retain many liver-specific functions (8–10), including the capacity to conjugate and excrete bilirubin (11).

Materials and Methods. MH_1C_1 cells and fibroblasts used in control experiments were grown in small plastic flasks in Ham's F10 medium (12) supplemented with 2.5% fetal calf serum and 15% horse serum in a humidified atmosphere of 5% CO_2 and 95% air. Two to six replicate cultures were studied at the same time; approximately half of these had been preincubated with phenobarbital (0.2 mg/ml) for 5 days. At the beginning of the experiment the medium was replaced with 1.5 ml of fresh medium containing either 35 μCi of glycine-2-^{14}C (18 mCi/mmole; New England Nuclear Corp.) or 4 μCi δ-aminolevulinic acid (ALA)-3,5-^{3}H (455 mCi/mmole; New England Nuclear Corp.). Incubations were terminated at 24 hr. The cells were washed with saline, and cell protein was measured by the method of Lowry *et al.* (13). Labeled bilirubin was crystallized (14) from the medium after addition of rat bile containing a measured amount of nonradioactive bilirubin. The pigment was recrystallized twice, and its specific activity was measured in a liquid scintillation spectrometer (14). Total bilirubin radioactivity in the medium was calculated from the specific activity of the crystallized pigment and the total amount of carrier added; the concentration of bilirubin present before addition of the carrier was too low to be measured. Excellent recovery was achieved with this technique when known amounts of conjugated bilirubin-^{14}C in rat bile or unconjugated bilirubin-^{14}C in dilute alkali were added to culture medium.

Results. MH_1C_1 cells incorporated an average of 0.013% of the glycine-^{14}C substrate into bilirubin-^{14}C/mg of cell protein (Table I). Phenobarbital did not induce an increase in cell protein, but led to a consistent small rise in labeled bilirubin production. No radioactivity was recovered in bilirubin crystallized from medium into the absence of cells; however, low levels were observed with fibroblast cultures. Incorporation of ALA-^{3}H into labeled bilirubin by MH_1C_1 cells (Table II) was seventyfold greater than with glycine-^{14}C; slight enhancement was observed in some but not all cultures that had been preincubated with phenobarbital. Unexpectedly, fibroblasts produced labeled bilirubin from ALA-^{3}H almost as effectively as the MH_1C_1 cells.

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TABLE I. Incorporation of Glycine-2-^{14}C into Bilirubin-^{14}C by Cells in Culture.[a]

Cell type	Treatment[b]	No. of expts.	Cell protein (mg)	% Glycine-^{14}C incorporated (per mg of protein)
MH_1C_1	None	4	0.99 ± 0.02	0.013 ± 0.001
	Phenobarbital	6	0.96 ± 0.02	0.017 ± 0.002
Fibroblasts	None	4	1.4 ± 0.3	0.001 ± 0
	Phenobarbital	2	2.0	0.001
			1.7	0.001
Medium only	None	4	—	0

[a] Cultures were incubated with 35 μCi of glycine-^{14}C for 24 hr, and cell protein and bilirubin radioactivity in the medium were then measured. Mean values ± SE are shown.

[b] Preincubation with phenobarbital, 0.2 mg/ml, for 5 days.

The absolute rate of bilirubin production could be estimated from the specific activity of labeled precursor in the medium and its rate of incorporation into bilirubin. In the experiments with glycine-^{14}C, MH_1C_1 cells not treated with phenobarbital produced an average of 0.03 mμmole of bilirubin/day/mg of cell protein. The mean value for the studies with ALA-^{3}H was 0.04. Assuming that the hepatic pigment fraction in living rats comprises 10–15% of total bilirubin formation (2), the corresponding value for rat liver *in situ* is 0.17–0.26 mμmole of bilirubin formed/day/mg of liver protein.

Discussion. MH_1C_1 cells are neoplastic parenchymal cells. These findings therefore are consistent with the concept that hepatocytes produce at least part of the nonerythroid bilirubin component. Moreover, since this pigment fraction appears to be derived from tissue hemes (5, 6, 15), MH_1C_1 cells must have the capacity both to synthesize heme and then convert it to bilirubin. The greater incorporation of ALA compared to glycine presumably reflects the presence in MH_1C_1 cells of ALA-synthetase, the rate-controlling enzyme in heme biosynthesis, and corresponds to the findings in isolated rat liver (4) and intact animals and man (1–3, 16). Indeed, the observed seventyfold difference between the incorporation of ALA-3, 5-^{3}H and that of glycine-2-^{14}C underestimates the preferential utilization of the former substrate for hepatic heme and bilirubin synthesis, since 87.5% of the ^{14}C but only 28.1% of the ^{3}H is potentially available for incorporation into bilirubin (14, 17).

It is of interest that the fibroblasts also

TABLE II. Incorporation of ALA-3,5-^{3}H into Bilirubin-^{3}H by Cells in Culture.[a]

Cell type	Treatment[b]	No. of expts.	Cell protein (mg)	% ALA-^{3}H incorporated (per mg of protein)
MH_1C_1	None	6	0.97 ± 0.04	0.91 ± 0.09
	Phenobarbital	7	0.98 ± 0.04	1.15 ± 0.17
Fibroblasts	None	2	1.8	0.98
			1.9	0.70
	Phenobarbital	2	1.5	0.72
			1.6	0.75
Medium only	None	4	—	0.01 ± 0.01[c]

[a] Experiments were performed as described in Table I except that ALA-^{3}H, 4 μCi, was used instead of glycine-^{14}C. Mean values ± SE are shown.

[b] Preincubation with phenobarbital, 0.2 mg/ml, for 5 days.

[c] Based on assumed cell protein of 1 mg.

formed labeled bilirubin from ALA-^{3}H, indicating that they contain many of the steps needed to synthesize and degrade heme. However, these cells appear to be deficient in ALA-synthetase since they fail to utilize glycine to any appreciable extent. In addition, unlike MH_1C_1 cells, these fibroblasts lack the capacity to conjugate bilirubin (11).

In contrast to the findings in living rats (6, 15, 18), phenobarbital caused only slight enhancement of bilirubin production by MH_1C_1 cells. We have observed a similar disparity with regard to bilirubin conjugation (11). Failure of stimulation might be explained by the neoplastic nature of these cells. Alternatively, the metabolic sources of the hepatic bilirubin fraction may already be maximally induced under the conditions of culture. It has been suggested that this pigment fraction may originate in large part from cytochromes in the microsomal fraction of liver, since the activity of these heme-containing enzymes and the size of the nonerythroid bilirubin component are both augmented in rats treated with phenobarbital (15). In view of the poor stimulation of labeled bilirubin production in MH_1C_1 cells, it will be of interest to determine whether phenobarbital leads to significant changes in the activity of enzymes such as cytochromes P-450 and b_5 in these cells. Through investigations such as these, this unique *in vitro* model should lead to further understanding of the processes of hepatic heme and bilirubin production.

Conclusions. A clonal strain (MH_1C_1) of rat hepatoma cells synthesized labeled bilirubin with either glycine-2-^{14}C or δ-aminolevulinic acid-3,5-^{3}H as precursor. In contrast to the findings in living rats, labeled bilirubin production is only slightly enhanced in MH_1C_1 cells that have been preincubated with phenobarbital. These studies indicate that hepatic parenchymal cells are capable of producing at least part of the nonerythroid fraction of bile pigment. Moreover, they provide a unique experimental model for the further study of hepatic heme and bilirubin metabolism.

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