METABOLISM OF BILIRUBIN BY A
CLONAL STRAIN OF RAT HEPATOMA CELLS

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ABSTRACT
These studies demonstrate that the MH1C1 strain of rat hepatoma cells has the ability to
take up and conjugate bilirubin and then excrete the conjugated pigment into the culture
medium. On incubation with unconjugated bilirubin, the average rate of appearance of
conjugated bilirubin in the medium was 4.4 ± 0.20 µg per mg of cell protein per hour
(mean ± se). The products formed from bilirubin by MH1C1 cells were chromatographi-
cally identical to those found in normal rat bile. Assay of bilirubin UDP-glucuronyl
transferase activity in homogenates of MH1C1 cells gave a value of 3.3 ± 0.50 µg of con-
jugated pigment formed per mg protein per hour, only moderately less than the enzyme
activity of liver from normal rats. Rat fibroblasts in culture did not conjugate bilirubin,
nor did they contain bilirubin UDP-glucuronyl transferase activity. As in living animals,
flavaspidic acid inhibited bilirubin metabolism by MH1C1 cells, suggesting that the mech-
anism for bilirubin uptake is similar to that of normal liver. In contrast to the findings in
animals, however, preincubation of MH1C1 cells with phenobarbital led to only minimal
enhancement of pigment conjugation. MH1C1 cells represent the first example of a clonal
strain of cells in culture in which many of the pathways of hepatic bilirubin metabolism
remain intact. They should, therefore, serve as a useful model for studies of bile pigment
metabolism which are not easily performed in the living animal.

INTRODUCTION
The movement of bilirubin from plasma to bile
involves several processes: uptake of unconjugated
bilirubin by liver cells, conjugation with glucuronic
acid, and secretion of the conjugated pigment
into the bile. Despite extensive investigation, the
mechanisms involved in each of these processes
are incompletely understood. Studies of bilirubin
metabolism in hepatic cells in culture would circum-
vent a number of difficulties encountered in experi-
ments with intact animals or with liver
slices or homogenates. However, this approach
has been hampered by the lack of a line of cells in
which bilirubin conjugation remains intact; the
few studies reported have been concerned with
cells incapable of conjugating bilirubin (1, 2).
We have, therefore, investigated bilirubin metab-
olism in a clonal strain of rat hepatoma cells
(MH1C1) which performs a number of liver-spe-
cific functions (3, 4). These cells produce serum
albumin (3), tyrosine aminotransferase (4), and
the ninth component of complement (4, 5). They
also conjugate testosterone with glucuronic acid
(4). Albumin production and the level of tyrosine
aminotransferase are both stimulated by treat-
ment with hydrocortisone (4, 6).
This report describes experiments showing
that MH1C1 cells contain the enzyme, bilirubin UDP-glucuronil transferase, and are capable of transforming unconjugated bilirubin to the conjugated form at rates comparable to those observed in the liver of intact rats.

MATERIALS AND METHODS

Materials

Unconjugated bilirubin was obtained from the Sigma Chemical Co., St. Louis, Mo. (Lot No. 88B-8110). A 50 mg/100 ml solution was made by dissolving 5 mg of bilirubin rapidly in 0.5 ml 0.05 N NaOH, followed by addition of 9.5 ml of a 2.4 g/100 ml solution of bovine serum albumin (Albumin Bovine Crystalline, Nutritional Biochemical Corp., Cleveland, Ohio, Control No. 8161). Fresh bilirubin solutions were prepared immediately before each experiment.

Flavaspidic acid-N-methylglucaminate was a gift from Dr. Esa Aho, Laaketehtdas Leiras, Turku, Finland.

Hydrocortisone sodium succinate was obtained from the Upjohn Company, Kalamazoo, Michigan. Stock solutions of 9 X 10^-4 M hydrocortisone were made in 0.15 M NaCl and stored at -20°C for no more than 1 wk.

Phenobarbital was obtained from Merck and Co., Inc., Rahway, New Jersey. It was dissolved freshly each day in serum-free growth medium at a concentration of 4 mg/ml.

Precoated silica gel plates (F-25, 0.25 mm) for thin-layer chromatography were obtained from Merck and Co., Inc. (see above).

Viokase was purchased from the Viobin Corporation, Monticello, Illinois.

Methods

METHODS OF CELL CULTURE: The cells used in these experiments were derived from the transplantable Morris rat hepatoma No. 7795. They were established as a clonal strain, MH1C1, by U. I. Richardson et al. (3), and have been serially propagated since 1967. Cells were grown in 75 sq cm (250 ml) Falcon plastic tissue culture flasks in Ham’s F 10 medium (7) supplemented with 15% horse serum and 2.5% fetal calf serum. A few experiments were also performed with Dulbecco’s modification of Eagle’s medium1 (8, 9) supplemented with 10% fetal calf serum and 7.5% horse serum. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Subcultures were made by incubating the cells with 0.1% Viokase solution in phosphate-buffered saline for 5-10 min at 37°C. The dispersed cells were then gently centrifuged and resuspended in fresh medium.

Control experiments were carried out both with medium in the absence of cells and with a clonal strain of rat fibroblasts (R5) grown under the same conditions as the MH1C1 cells. Cell protein was determined by the method of Lowry et al. (10).

MEASUREMENT OF BILIRUBIN CONJUGATION BY CELLS IN CULTURE.2 At the beginning of each experiment the growth medium was removed and fresh prewarmed medium was added. Bilirubin solution was then added to give a final bilirubin concentration of 1.25-5.0 mg/100 ml in a total incubation volume of 6.0 ml. All flasks were prepared in duplicate. Cultures were incubated at 37°C, and 0.25 ml samples were removed at intervals (see Results) for immediate measurement of the concentration of conjugated bilirubin.

Conjugated bilirubin was measured as the diazonium salt of ethyl anthranilate, as described by VanRoy and Heirwegh (11). In one experiment, conjugated bilirubin was also measured according to the method of Weber and Schalm (12); similar results were obtained with both assays. Conjugated bilirubin was further identified in two thin-layer chromatographic systems. In the first system,3 native bile pigments were isolated after precipitation of proteins with a 1:1 solution of saturated (NH4)2SO4: absolute ethanol. The precipitate was washed several times with a 1:1 solution of methanol:chloroform, and the combined supernatant fractions were evaporated to dryness in vacuo. The residue was taken up in methanol:chloroform, applied to silica gel plates, and developed in chloroform: methanol: acetic acid (8.5:1.5:0.3). In this system, unconjugated bilirubin travels with the solvent front; conjugated pigment I (13) moves with an Rf of about 0.15; and conjugated pigment II (13) moves just above the origin. The diazo-derivatives were extracted into ethyl acetate, and the latter was washed with water and then evaporated in vacuo. The pigments were separated on silica gel plates in a solvent system of isooctylacetate: propionic acid: N-propanol: water (4:3:2:1). The diazo-derivative of unconjugated bilirubin traveled

1 As prepared for Dr. Michael Oxman by Microbiological Associates, Inc., Bethesda, Maryland.

2 Although the term "bilirubin conjugation" is used for convenience, the appearance of conjugated bilirubin in the medium actually involves several processes: uptake of unconjugated bilirubin by the cells, conjugation with glucuronic acid, and release of the conjugated pigment into the medium.

3 Based on a personal communication from Dr. K. P. M. Heirwegh.
just behind the solvent front, while that of conjugated bilirubin had an Rf value of 0.55-0.60. Pigment I isolated from the first chromatographic system yielded equal quantities of both azo pigments (13); pigment II yielded only the conjugated product (13).

**ASSAY OF BILIRUBIN UDP-GlcURONYL TRANSFERASE:** In each experiment, the contents of several identical bottles of cells (a total of 4.2-14.7 mg cell protein) were pooled for enzyme assay. The cells were recovered from the culture vessels by incubation with Viokase. They were then centrifuged, washed once, resuspended in a solution of 0.25 M sucrose and 0.001 M EDTA, and frozen at -70°C until assay, which was usually performed 2-4 days later. Glucuronyl transferase activity was measured by the method of VanRoy and Heirwegh (11), as modified by Black and Billing (15).

**INCUBATION WITH DRUGS:** In some experiments, either $3 \times 10^{-4}$ M hydrocortisone or $9.6 \times 10^{-4}$ M phenobarbital was included in the culture medium. Medium was changed every day, with addition of fresh hydrocortisone or phenobarbital. Formation of conjugated bilirubin by cells in culture was measured both during 6-hr incubations with drug and after 8 days of pretreatment with drug. Control measurements were performed with equivalent quantities of cells from the same subculture which had not been treated with hydrocortisone or phenobarbital. In addition, glucuronyl transferase activity in cell homogenates was measured after 8 days of pretreatment with either drug.

In another experiment, flavaspidic acid-N-methylglucaminate was incorporated into the medium in final concentrations of 1 and 5 mg/100 ml, and the formation of conjugated bilirubin was measured over the ensuing 24 hr. Control measurements were performed in replicate flasks to which no drug had been added.

**RESULTS**

**Formation of Conjugated Bilirubin by MH1C1 Cells in Culture**

The rate of appearance of conjugated bilirubin in culture medium is shown in Figs. 1 and 2. Formation of conjugate occurred at a constant rate over the first 6-8 hr of incubation with bilirubin (Fig. 1). After this period, the rate of appearance of conjugated bilirubin began to decrease (Fig. 2). No conjugate was identified when unconjugated bilirubin was incubated with either rat fibroblasts or medium in the absence of cells.

The decreasing rate of conjugation illustrated in Fig. 2 is doubtless explained in part by depletion of bilirubin substrate during prolonged incubation with MH1C1 cells. In addition, instability of unconjugated and/or conjugated bilirubin under the conditions of culture may have played a role. No significant deterioration of unconjugated pigment was observed during a 24 hr incubation in the absence of cells. However, when medium from a 30 hr incubation with MH1C1 cells was incubated for an additional 24 hr in the absence of cells, the concentration of conjugated bilirubin decreased at a rate of 2-3% per hour. Thus, the amounts of conjugate measured during prolonged incubation periods are minimal estimates of the
amounts that can actually be conjugated by these cells.

The formation of conjugate varied directly with the concentration of unconjugated bilirubin in the incubation medium over a dose range from 1.25 to 5.0 mg/100 ml unconjugated bilirubin (Fig. 2). At each of these concentrations, approximately 50% of the total substrate was conjugated in 30–40 hr. The amount of conjugate formed did not increase significantly with longer incubation times, or with further increases in substrate concentration. Indeed, at bilirubin concentrations of 10 mg/100 ml, MH1C1 cells produced only small quantities of conjugated bilirubin and showed morphological signs of cellular injury when examined by phase-contrast microscopy. Since the cytotoxicity might have been due to excess bilirubin unbound to albumin, an experiment was performed in which bilirubin was dissolved in a solution containing more than twice the concentration of bovine serum albumin than was routinely employed. Under these conditions, MH1C1 cells did not show morphological signs of injury with bilirubin concentrations of 10 mg/100 ml, although they did at 15 mg/100 ml. However, with the increase in bilirubin concentration to 10 mg/100 ml the formation of conjugated bilirubin was no higher than with substrate concentrations of 5 mg/100 ml. Therefore, under the conditions of these experiments, bilirubin conjugation by MH1C1 cells in culture appeared to be maximal with a bilirubin concentration of 5 mg/100 ml. In all subsequent experiments, bilirubin conjugation was assessed in the presence of 5 mg/100 ml of bilirubin during the first 6 hr of incubation, i.e. during the linear portion of the curve (Fig. 1). Under these conditions, the rate of formation of conjugated bilirubin in 22 consecutive experiments varied from 2.4 to 7.1 µg of conjugated bilirubin per mg cell protein per hour, with a mean value of 4.4.

The conjugated pigment formed by MH1C1 cells was further characterized by chromatographic analysis, with the pigments in normal rat bile as a standard of comparison. Fig. 3 shows the results of thin-layer chromatography of both the native bile pigments and their diazo-derivatives isolated from medium that had been incubated with MH1C1 cells for 30 hr. Bands corresponding to conjugated bilirubin (pigments I and II) and to the diazo-derivative of conjugated bilirubin were readily identified. The relatively large quantities of unconjugated bilirubin and the diazo-derivative of unconjugated bilirubin in the culture medium as compared to rat bile are attributable to the excess of unconjugated bilirubin substrate in the medium.

**Effect of Drugs on Bilirubin Conjugation by Cells in Culture**

When incubated with MH1C1 cells for 6 hr, neither phenobarbital nor hydrocortisone significantly affected bilirubin conjugation, although the former may have had a small enhancing effect (Table I). Similarly, there was no change in cell protein in these short-term incubations. In cells pretreated with phenobarbital for 8 days, cell
Figure 4 Effect of flavaspidic acid-N-methylglucaminate on bilirubin metabolism by MH1C1 cells in culture. The drug together with 5 mg/100 ml of unconjugated bilirubin was added to medium at zero time. Measurements were made as described in Fig. 2.

Protein remained unchanged, but there was a small increase in the capacity to conjugate bilirubin (Table I). After pretreatment with hydrocortisone for 8 days, there was a decrease in cell protein. Thus, although the total formation of conjugated bilirubin diminished slightly, there was actually an increase in bilirubin conjugation per mg cell protein in these cultures (Table I).

As shown in Fig. 4, flavaspidic acid at a concentration of 5 mg/100 ml reduced the formation of conjugated bilirubin to less than 25% of that in control cultures. At a concentration of 1 mg/100 ml, a smaller effect was noted. Flavaspidic acid (5 mg/100 ml) had no inhibitory effect on serum albumin production by MH1C1 cells, indicating that the suppression of bilirubin metabolism was not due to a nonspecific toxic effect of the agent.

### Table I

| Treatment          | 6 hr incubation with drug* | 8 days pretreatment with drug‡ |
|--------------------|----------------------------|--------------------------------|
|                    | Cell protein (µg/mg protein/hr) | Conjugated bilirubin (µg/mg protein/hr) | Cell protein (µg/mg protein/hr) | Conjugated bilirubin (µg/mg protein/hr) |
| Control            | 2.2 | 6.2 | 2.8 | 2.4 | 6.9 | 2.9 |
|                    | 2.3 | 7.6 | 3.3 | 2.4 | 7.6 | 3.2 |
| Phenobarbital      | 2.2 | 7.9 | 3.6 | 2.4 | 9.6 | 4.0 |
|                    | 2.2 | 8.1 | 3.7 | 2.7 | 8.5 | 3.2 |
| Hydrocortisone     | 2.1 | 6.2 | 2.9 | 1.4 | 5.6 | 4.1 |
|                    | 2.3 | 8.1 | 3.5 | 1.3 | 6.6 | 4.9 |

* The drug was present in the medium together with unconjugated bilirubin during the 6 hr incubation.
‡ The drug was not present during the 6 hr incubation with unconjugated bilirubin.
§ The concentration of unconjugated bilirubin in the medium was 5 mg/100 ml. All flasks were replicated from the same subculture. Each value given is the result from a single flask.

Enzyme activity in 14 separate pools of cells varied from 1.2 to 7.6 µg of conjugated bilirubin formed per mg cell protein per hour, with a mean value of 3.3. This value is in good agreement with bilirubin conjugation by cells in culture (4.4 ± 0.20 µg of conjugated bilirubin/mg cell protein/hr, mean ± SE). Rat fibroblasts had no detectable bilirubin glucuronyl transferase activity.

Pretreatment with phenobarbital for 8 days did not lead to an increase in bilirubin glucuronyl transferase activity (Table II). After 8 days of pretreatment with hydrocortisone (Table III), there was a decrease in cell protein and a rise in bilirubin conjugation per mg cell protein, as had been observed in the experiments with intact cells (Table I).

**Discussion**

These experiments provide the first evidence for bilirubin conjugation by cells in culture. On incubation with unconjugated bilirubin, the MH1C1 strain of rat hepatoma cells produced conjugated pigments identical with those found in normal rat bile using two different thin-layer chromatographic systems (Fig. 3). Moreover, bilirubin UDP-glucuronyl transferase activity was demonstrated in homogenates of MH1C1.
TABLE II
Effects of Phenobarbital on Bilirubin UDP-Glucuronyl Transferase Activity in MH1C1 Cells

| Exp. No. | Control | Pheno-barbital | Control | Pheno-barbital |
|---------|---------|----------------|---------|----------------|
|         | mg      | µg conj. bili./mg protein/hr | mg      | µg conj. bili./mg protein/hr |
| 1       | 10.5    | 1.5            | 11.5    | 1.5            |
| 2       | 5.5     | 3.1            | 4.0     | 2.9            |
| 3       | 13.0    | 1.3            | 14.5    | 1.6            |

Cultures were treated with phenobarbital (9.6 × 10⁻⁴ M) for 8 days. Cells from six to eight replicate flasks were pooled, washed, and transferase activity was determined on the homogenates. Control cells were handled in the same manner except that they were not treated with phenobarbital. Each value is the mean of duplicate determinations.

TABLE III
Effects of Hydrocortisone on Bilirubin UDP-Glucuronyl Transferase Activity in MH1C1 Cells

| Exp. No. | Control | Hydrocortisone | Control | Hydrocortisone |
|---------|---------|----------------|---------|----------------|
|         | mg      | µg conj. bili./mg protein/hr | mg      | µg conj. bili./mg protein/hr |
| 1       | 1.0     | 5.3            | 0.8     | 7.7            |
| 2       | 1.0     | 4.1            | 0.5     | 7.8            |
| 3       | 4.4     | 1.2            | 2.9     | 4.1            |

Cultures were treated with hydrocortisone (3 × 10⁻¹ M) for 8 days. Cells from six to eight replicate flasks were pooled, washed, and transferase activity was determined on the homogenates. Control cells were handled in the same manner except that they were not treated with hydrocortisone. Each value is the mean of duplicate determinations.

Cultures were treated with phenobarbital (9.6 × 10⁻⁴ M) for 8 days. Cells from six to eight replicate flasks were pooled, washed, and transferase activity was determined on the homogenates. Control cells were handled in the same manner except that they were not treated with phenobarbital. Each value is the mean of duplicate determinations.

Cultures were treated with hydrocortisone (3 × 10⁻¹ M) for 8 days. Cells from six to eight replicate flasks were pooled, washed, and transferase activity was determined on the homogenates. Control cells were handled in the same manner except that they were not treated with hydrocortisone. Each value is the mean of duplicate determinations.

cells, and the activity was comparable to that found in normal rat liver. Enzyme activity in MH1C1 cells varied from 1.2 to 7.6 µg of bilirubin conjugated per mg of cell protein per hour; measured in the same way, bilirubin glucuronyl transferase activity in homogenates of liver from five Sprague-Dawley rats ranged from 3.1 to 8.4 µg per mg of liver protein per hour (Robinson, S. H., unpublished data).

The conversion of unconjugated to conjugated bilirubin by MH1C1 cells demonstrates the presence of pathways for bilirubin uptake and secretion as well as conjugation in these cells. Although several mechanisms have been proposed for the inhibition of bilirubin excretion by flavaspidic acid (17), interference with hepatic uptake seems the most likely (16). The inhibition observed when MH1C1 cells were incubated with flavaspidic acid (Fig. 4), therefore, suggests that the uptake process in MH1C1 cells may be similar to that in normal liver. Since flavaspidic acid has been shown to compete with bilirubin for binding to small cytoplasmic proteins which may play a role in this metabolic process (18), the present findings further suggest that these proteins are also present in the cultured cells.

Although the normal mechanism for secretion of conjugated bilirubin may also be intact in MH1C1 cells, the conditions of dispersed cell culture make it difficult to evaluate this aspect of the excretory process. In intact rats, secretion of conjugated bilirubin into the bile is the rate-limiting step in hepatic bilirubin excretion (19-21). The maximum rate of secretion of conjugated pigment into the bile in vivo is substantially lower than the capacity for bilirubin conjugation (22), and conjugated pigment that cannot gain access to the bile is "regurgitated" back into the plasma (19, 21, 22). With MH1C1 cells, on the other hand, the rate of appearance of conjugated bilirubin in the medium is similar to the rate of conjugation by cell homogenates. It seems possible that the egress of conjugated bilirubin from the cells in culture may occur by more than one pathway, including both secretion and "regurgitation," without the anatomic diversion into bile and plasma found in liver cells in vivo. Under the conditions of cell culture, therefore, conjugation rather than secretion might become the limiting step in bilirubin excretion.

A variety of conditions affected bilirubin metabolism by MH1C1 cells. The rate of appearance of conjugated bilirubin in the medium varied with the substrate concentration, and maximal rates were observed at a bilirubin concentration of 5 mg/100 ml (Fig. 2). Although this may indicate that the amount of substrate limits the rate of conjugation, the complexity of the system makes interpretation uncertain. Higher substrate concentrations were associated with morphological evidence of cell damage, and it is possible that more subtle changes may have occurred in some of the other experiments. In addition, the binding
affinity of albumin for bilirubin probably plays a
time in governing the rate of pigment uptake by
liver cells. Since this affinity varies with the species
from which the albumin is derived (23, 24), it is
possible that the use of bovine rather than rat
albumin as a vehicle may have affected the results.

Phenobarbital treatment of intact animals leads
to a significant rise in the capacity to conjugate
bilirubin (22, 25-27). In the present experiments,
however, there was little or no enhancement of
either enzyme activity in cell homogenates or
bilirubin conjugation by cultured cells after 8 days
of treatment with this drug (Tables I and II). It
is possible that stimulation did not occur because
the MH1C1 cells are neoplastic and have lost their
responsiveness to this agent. Alternatively, enzyme
activity may already be maximal under the condi-
tions of culture.

The specific activity of bilirubin glucuronyl
transferase did increase after hydrocortisone treat-
ment (Tables I and III). However, this
enhancement was more apparent than real, and
resulted primarily from a decrease in cell protein.
As shown in Table I, total bilirubin conjugation
by cells in culture actually decreased somewhat
after prolonged treatment with hydrocortisone.
These findings are consistent with the hypothesis
that, as compared to the soluble proteins, a mem-
brane-bound enzyme such as glucuronyl transferase is relatively little affected by the anti-
anabolic effect of hydrocortisone.

These experiments demonstrate both similarities and
differences between MH1C1 cells in culture
and normal rat liver with regard to bilirubin
metabolism. Pigment uptake and conjugation
are similar in many respects in these two systems,
although there is but little enhancement of bili-
rubin conjugation by phenobarbital in MH1C1
cells. Secretion of conjugated bilirubin may also
be intact, but may be obscured by additional
routes of bilirubin egress from the cultured cells.
In addition, preliminary experiments indicate
that MH1C1 cells synthesize labeled bilirubin
from both glycine-2,3,5-3H and delta-aminolevulinic
acid-3,5-3H, demonstrating that these cells also
contain the as yet unidentified heme sources of the
hepatic fraction of early-labeled bilirubin (28).
For these reasons and because they are a clonal
strain of hepatic parenchymal cells whose environ-
ment can be modified according to experimental
design, MH1C1 cells should prove of great value
in future studies of hepatic bilirubin metabolism.

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