PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 918–924 (1974)

The Conjugation of Bilirubin by Rat Hepatoma Cells in Tissue Culture[1] (37924)

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Attempts to demonstrate bilirubin conjugation by cells in culture were unsuccessful (1, 2) until the work of Rugstad *et al.* (3) with the MH_1C_1 clonal strain of rat hepatoma cells. Glucuronyl transferase activity per mg cell protein in those cells was similar to that in liver from normal rats, and it was shown that the cells were able to conjugate bilirubin and synthesize it from glycine and Δ-aminolevulinic acid (4). The effect of albumin binding on conjugation and toxicity of bilirubin was also studied in that strain (5). We similarly examined for evidence of bilirubin conjugation a series of 28 clonal cell lines isolated in this laboratory from rat, chimpanzee, and human liver tissue, including two from human hepatomas, as well as eight clones isolated from Chang's liver line, and none was found. A culture of rat hepatoma cells derived from the Reuber hepatoma H-35 strain H4-II-E did, however, give evidence of conjugation. This report described experiments which illustrate that that strain has an ability to take up and conjugate bilirubin and excrete the conjugated pigment into the medium in a manner and at rates similar to the MH_1C_1 strain, and which illustrate the effect of various conditions of growth on conjugating capacity. Clones isolated from the competent strain were then also studied, and their conjugating capacities were compared with the parent culture.

In 1961, M. D. Reuber described a bile-secreting, transplantable hepatocellular carcinoma in the rat (6). An explanted mince of this tumor was cultured (7) and after a single reinoculation into AxC strain rats, the resultant tumor was again explanted to culture. Colonies with epithelial morphology were noted and picked from a fibroblastic monolayer, and after repeated colony separations, an epithelial cell strain was established. Attempts at single cell cloning were unsuccessful, but the strain was free of fibroblasts and produced a typical Reuber H-35 type tumor on inoculation *in vivo*. The resultant strain H4-II-E exhibited corticosteroid induction of tyrosine amino transferase, contained the hepatic enzymes histidase, ornithine transaminase, tyrosine transaminase, thymine reductase, and glucokinase and had a modal chromosome count of 46. A recent chromosome analysis of the cells maintained in culture for 11 years yielded a mean count of 48, with numerous abnormal (marker) chromosomes (8).

In 1965 Arias (9) reported glucuronyl transferase activity in the Reuber H-35 hepatoma to be 80% of the level in host liver. A more recent study of Reuber H-35 hepatoma tissue homogenates from AxC Irish rats (10) using detergents to activate membrane-bound glucuronyl transferase showed the enzyme to be increased relative to normal or host liver when compared on a tissue-protein basis and assayed with *p*-nitrophenol as acceptor.

Materials and Methods. The cells used

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were provided by Dr. Carmia Borek, Institute for Cancer Research, Columbia University, New York (8) and are the minimal deviation hepatoma cell line H4-II-E (7) derived from the Reuber hepatoma (6). They are referred to in this report as H4. They have been maintained *in vitro* for 11–12 years.

Clones were isolated from the H4 cultures by methods similar to those of Coon (11), as previously described (12), and stored in liquid nitrogen. Clones included in this study were designated H4-1, -2, -3, -4, -5, -7, -8, -9, -11, -12, -13, -14, -15, -16, -17, -23, and -24. Clonal cultures were grown from 17 to 99 days after thawing and passed one or more times before metabolic studies were made. The parent H4 culture, similarly stored, showed no difference in bilirubin metabolism 5, 14, or more than 100 days post-thawing.

The MH_1C_1 clonal strain of rat hepatoma cells was obtained from the American Type Culture Collection, Rockville, MD. Non-liver cell lines included in the study were HeLa, a recently isolated normal human skin fibroblast culture provided by Dr. J. German, Laboratory of Human Genetics, The New York Blood Center, and a clonal strain of rat kidney fibroblast isolated in this laboratory (Cl 135).

The culture medium was a modification of Ham's F 12 medium (13) designated F 12K (14) and contained antibiotics as previously described (12).

Fetal calf serum (FCS) was obtained from Grand Island Biological Company. The albumin content of the serum was 2.3 g/100 ml measured by an automated total protein technique (15) based on the biuret reaction (16) and cellulose acetate electrophoresis (17). The total protein was 3.8 g/100 ml.

Unconjugated crystalline bilirubin, Type B 4126, was obtained from Sigma Chemical Company, St. Louis, MO. Bovine albumin (Bovine Albumin Powder-fraction V from bovine plasma) was obtained from Armour Pharmaceutical Co., Chicago, IL. A 40 mg/100 ml bilirubin in saline stock solution stabilized with bovine albumin was sterilized by sequential filtration (Millipore Filter Corp., Bedford, MA). A mixture of 15 ml F 12K medium and 5 ml FCS was combined with stock solution to yield a final bilirubin concentration of the desired level in the nutrient mixture, usually 5 mg/100 ml. The bilirubin to total albumin molar ratio was approximately 0.75/1.0. Fresh solutions were prepared immediately before each experiment.

Cell culture methods. Cultures for conjugation of adequate amounts of bilirubin for chromatographic identification by paper chromatography were grown in 670 cm^2 Bellco cell production roller vessels (Bellco Glass Inc., Vineland, NJ) equilibrated with 5% carbon dioxide in air before sealing and incubation.

For all other experiments cells were grown in Falcon plastic culture dishes 100 × 20 mm in F 12K medium supplemented with 5% FCS (17% for MH_1C_1 cultures) and were incubated at 36.5° in humidified 95% air with 5% carbon dioxide. Cells were removed from plates for subculture or prepared for cell protein assay by incubating cultures with 0.25% trypsin in Moscona balanced salt solution (18) for 10 min at 37°. They were centrifuged, resuspended in fresh medium, and replated at 7×10^5 to 5×10^6 cells per plate or approximately 1×10^7 cells per bottle. Cultures were fed three times per week and studied upon reaching confluent growth, a period of 7 days after the last replating in culture dishes, and 9 days in bottle culture. Cell protein was measured by the method of Lowry *et al.* (19). An inverted biological microscope equipped for phase contrast (Wild M40 tissue culture microscope) was used to observe cell morphology.

Identification and rate of formation of conjugated bilirubin. Bilirubin nutrient mixture (40 ml) was incubated with cultures having 18–20 mg cell protein per bottle for 48 hr, and was harvested and treated with diazotized sulfanilic acid (20). A control incubation without cells was similarly made. An *n*-butanol extract was washed with saline, evaporated to dryness, and the residue taken up in 1 *M* acetic acid for paper chromatography. Chromatography

standards of azobiliurbin pigments A and B were prepared from human gall bladder bile obtained at autopsy. The conjugated bilirubin derivative (azo B) of diazosulfanilic acid was isolated as outlined by Schachter (21), further purified by ascending paper chromatography using the solvent system of Schmid (22), and was converted to the unconjugated derivative (azo A) by alkaline hydrolysis. Another azo A standard was prepared from a chloroform solution of reagent unconjugated bilirubin.

Conjugating capacity was measured using plate cultures in which medium was replaced at the start of each experiment by 2.3 ml of bilirubin nutrient mixture. Duplicate plates of the H4 parent culture were studied at three bilirubin concentrations (3.2, 5.0, and 8.7 mg/100 ml), and the medium examined after incubation periods of from 6–30 hr. Following definition of the effect of incubation time and bilirubin concentration on the H4 parent culture's conjugating capacity, all subsequent measurements were made after 6 hr of incubation and at 5.1 mg% ($\pm$0.2 SD) initial bilirubin concentration, with bilirubin/albumin molar ratio 0.75/1.0. In each experiment with clonal cultures, two to four plates of the H4 parent culture were included as controls. Duplicate plates were made of H4 clonal cultures to be tested as well as skin fibroblast, rat kidney fibroblast, and HeLa cell controls. Cell morphology was examined before and after incubations with bilirubin nutrient mixture.

Conjugated bilirubin was measured on 0.25-ml specimens by the method of Van Roy and Heirwegh (23) and confirmed in two experiments by the method of Weber and Schalm (24). Total bilirubin was measured in 0.20-ml specimens by the method of Evelyn and Malloy (20).

Results. Identification of conjugated bilirubin. The diazosulfanilic acid derivative of bilirubin isolated from medium incubated with cells was identical with the azobilirubin pigment B standard (R_f 0.3–0.4) when compared by paper chromatography using Whatman 3 mm paper and the methyl ethyl ketone/propionic acid/water solvent system of Schmid (22) and by visible light spectrum. Control incubations in the absence of cells yielded no such pigment. The conjugated bilirubin concentration in the cell incubation medium after 48 hr was 1.7 mg/100 ml.

Effect of initial bilirubin concentration and incubation time on conjugating capacity. Having established the presence of conjugated bilirubin, the conjugating capacity was measured at three initial bilirubin concentrations as shown in Fig. 1 and found to increase with concentration up to 5 mg/100 ml. At a higher concentration, however, cell death and morphologic evidence of injury were seen and conjugating capacity was diminished. The cells depleted the medium of unconjugated bilirubin after 9–10 hr incubation as illustrated in Fig. 2, where the data at 5 mg/100 ml initial bilirubin concentration is presented in terms of the amount of bilirubin in the conjugated and unconjugated forms, based on serial measurements of total and conjugated bilirubin in the medium in a typical culture dish. The decrease in rate of conjugation after the initial constant rate corresponds with a marked depletion of available unconjugated bilirubin in the medium, due partly to conversion to the conjugated form as shown, but also probably due to bilirubin retained inside the cells and therefore undetected. There may also have occurred a small amount of degradation of the unconjugated

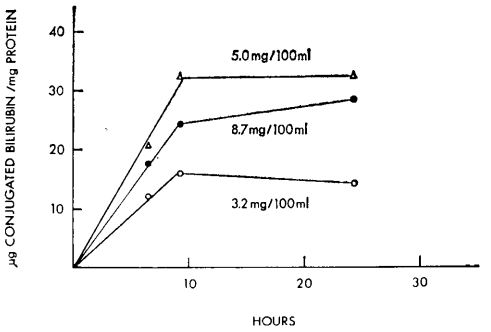

FIG. 1. The effect of initial unconjugated bilirubin concentration and duration of incubation on the rate of conjugated bilirubin appearance in the medium with H4 cells. Duplicate plates at each concentration, 3.2, 5.0, and 8.7 mg/100 ml, were each sampled alternately to yield four measurements at each concentration.

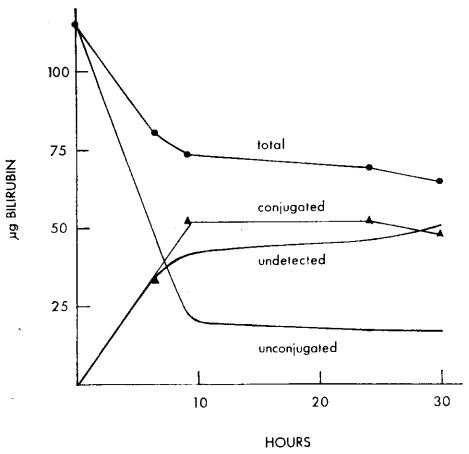

FIG. 2. The distribution of bilirubin in a typical dish culture experiment with H4 cells having cell protein content of 1.6 mg and provided with bilirubin nutrient mixture at the start of the experiment having unconjugated bilirubin content, 115 μg, and concentration, 5 mg/100 ml. Measured values for total bilirubin (●) and conjugated bilirubin (▲) in the medium are shown. Calculated values for unconjugated bilirubin in the medium and bilirubin lost to measurement (as by uptake into the cells) and thus undetected are derived from the measured values by assuming the initial 115 μg to be redistributed without degradation.

and conjugated forms to species not detected by either assay. The apparent unconjugated bilirubin concentration in the nutrient mixture incubated in the absence of cells decreased 0.02 mg/100 ml (0.4%) per hour during an interval equivalent to the experiment. The stability of the conjugated pigment was also measured. At the conclusion of an incubation with cells, medium containing 3.8 mg/100 ml total bilirubin and 2.2 mg/100 ml conjugated bilirubin was removed from the cultures and incubated in the absence of cells for 24 hr. The total bilirubin concentration again decreased at the rate of 0.02 mg/100 ml per hour, but the conjugate present decreased much more rapidly at the rate of 0.1–0.2 mg/100 ml per hour (4–8%), apparently reverting to the unconjugated form, since it was still detected by the total bilirubin measurement. This suggests that during a prolonged incubation of this type a portion of the bilirubin is conjugated, degraded to the unconjugated form, and may then be reconjugated one or more times. The actual rate of conjugation would therefore be somewhat higher than indicated by the data of Fig. 1.

Subsequent experiments were performed at 5 mg/100 ml initial bilirubin concentration, and the medium was sampled after 6 hr so as to avoid conditions where bilirubin substrate was limiting and the conjugation rate nonlinear, and to avoid the toxic effects of higher concentrations.

Similar incubations of bilirubin nutrient mixture with confluent cultures of HeLa, rat kidney fibroblast, and human skin fibroblast cells resulted in no detectable conjugated bilirubin in the medium. However, the total bilirubin present in the medium did decline during the initial 6 hr of those incubations by 22–28%. It was also noted that those cells, unlike the gray-white H4 cells, when rinsed free of medium and harvested at the conclusion of the incubation, were distinctly yellow in appearance, implying bilirubin uptake by or attachment to the cells without subsequent excretion into the medium. The MH_1C_1 cultures conjugated bilirubin in two cultures at the rate of 3.6 and 3.3 μg/mg/hr at 1.34 mg protein per plate.

Relationship between H4 cell protein content and bilirubin conjugating capacity. It was also noted that the specific conjugating capacity of the H4 cell cultures (expressed as micrograms conjugate formed per milligram cell protein per hour) decreased slightly with increased amount of cell protein present at the time of measurement. Most cultures were studied at or near to confluent growth as determined visually, and protein contents ranged from 1.41 to 2.46 mg/plate. In a single pair of cultures allowed to continue to grow for 14 days after reaching confluence, increasing the number of cells present, an increased nucleocytoplasmic ratio was observed, and the protein content reached 3.70–4.00 mg/plate. The relationship between conjugating capacity (micrograms conjugate formed per hour) and cell protein content (milligrams protein) for all 17 H4 cell parent cultures

over the range from 1.41 to 4.00 mg was established by a linear regression analysis (correlation coefficient $r = 0.90$). The slope of the regression line defined a decrease in specific conjugating capacity of from 3.3 μg/mg/hr at 1.4 mg protein/plate to 2.6 μg/mg/hr at 4.0 mg/plate.

Comparison of bilirubin conjugating capacity of H4 cell clones and H4 parent culture. Seventeen clones were isolated from the parent culture in order to determine whether the population included any cells unable to conjugate bilirubin and to assess the variability of conjugating capacity in the population. All were able to conjugate and had either similar or increased capacities when compared with the parent culture.

Values of micrograms conjugate per milligram cell protein per hour during the first 6 hr of incubation measured for 2–4 plates of each clone examined were compared with the H4 parent culture plates performance at various levels of cell protein per plate.

The selected high conjugating rate group of five clones comprised of H4-3, -4, -5, -12, and -15, conjugated at rates from 6.3 to 4.1 μg/mg/hr (mean 4.8 μg/mg/hr, $N = 15$) over the range of cell protein per plate of from 1.22 to 2.32 mg/plate. In this same range, the probable parent culture H4 rates as determined from the linear regression analysis line were 3.5 and 2.9 μg/mg/hr, respectively, and the maximum rate actually measured for any parent culture plate in this range was one observation at 4.2 μg/mg/hr at 1.41 mg/plate. This selected group of five clones with a high rate of conjugation thus differed from the parent culture by this quantitative measure.

Furthermore, even if all clonal culture experiments (all 17 clones with both high and low conjugating rates) are compared with the parent culture group of experiments, the mean of the clones 4.1 ± 0.8 μg/mg/hr (mean ± SD, $N = 43$) differs from the parent culture group mean of 3.0 ± 0.5 μg/mg/hr ($N = 17$), and the difference is significant at the 1% level.

Discussion. Excretion of conjugated bilirubin by H4 cultures which had been given unconjugated bilirubin implies that these cells have the biochemical mechanisms for bilirubin uptake, transport, conjugation, and excretion. This finding and similar observations on the MH_1C_1 rat hepatoma clonal cell line previously reported (3) and confirmed in this study suggest that this capability may not be unusual in cultured cells derived from liver tumors which appear to secrete bile when examined histologically (6). On the other hand, there have been no reported instances of bilirubin conjugation by normal euploid, nontumorigenic cell lines, even epithelioid lines of liver origin. In other unreported studies from this laboratory we have found no evidence of bilirubin conjugation in 17 rat liver, 2 human hepatoma, 7 human fetal liver, 2 chimpanzee liver, and 8 subclones isolated from Chang's liver line, even though the normal cell lines in this group include several which have been demonstrated to possess some liver-specific properties (12), for example, albumin synthesis.

When 17 clones, isolated from the H4 line, were examined for conjugating activity, no negative clones were found. In fact, although the clones varied in activity, none was less active than the parent culture. This may be due to a selective detoxifying enzyme system identical or related to glucuronyl transferase (EC 2.4.1.17). For example, it has been shown that the rabbit liver enzyme can conjugate a wide range of potentially toxic phenols, alcohols, amines, and fatty acids (25) and that Chinese hamster ovary cells are extremely sensitive to above optimal levels of linoleic acid (26). This kind of selective pressure, although more stringent in clonal culture, could also explain retention of the conjugating activity during repeated passage of the parent culture.

The slight decrease in specific conjugating capacity suggested by the data at higher cell protein contents, especially in postconfluent monolayer H4 cultures in which the cells have an apparent high nuclear/cytoplasmic ratio, may reflect their presumed higher proportion of nuclear to cytoplasmic components. The glucuronylating

enzyme is known to be associated with the smooth endoplasmic reticulum (27).

Established cell lines such as the MH_1C_1 and H4 can not only provide a convenient system for study of conjugation as has been shown, but can be expected to synthesize useful materials such as blood coagulation factors (28) and to serve as a source of particular enzyme-mediated functions to correct genetic defects (29).

Summary. The rat hepatoma cell strain H4-II-E, derived from the Reuber hepatoma H-35 and maintained *in vitro* for 12 years, takes up and conjugates bilirubin and excretes it into the culture medium at the rate of from 2.6 to 3.3 μg/mg cell protein/hr when supplied with unconjugated bilirubin at 5 mg/100 ml and a bilirubin/albumin molar ratio of 0.75. The conjugate identity was confirmed by comparison with conjugated bilirubin from human gall bladder bile by paper chromatography. HeLa cells, rat and human fibroblasts did not conjugate bilirubin. Seventeen clones were isolated from the parent culture, and all conjugated bilirubin. The mean conjugating rate of all clonal cultures was 4.1 ± 0.8 μg/mg/hr (mean ± SD, $N = 43$), compared with 3.0 ± 0.5 μg/mg/hr ($N = 17$) for the parent culture, a significant difference at the 1% level, possibly reflecting a selective effect of the cloning process for high conjugating capacity. This cell line and its subclones represent new examples of strains of cells in culture which metabolize bilirubin.

Determination of fetal calf serum albumin content by Dr. Roy W. Bonsnes, Director, Laboratory for Clinical Chemistry, The New York Hospital, is gratefully acknowledged.

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Received Oct. 22, 1973. P.S.E.B.M., 1974, Vol. 145.