Effect of Fibroblasts, Chang and Rat Liver Cells on Bilirubin in Tissue Culture.* (30350)

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We have described the effect of chick embryo macrophages on bilirubin introduced into tissue culture medium(1). The results of our study indicated that bilirubin was adsorbed onto the macrophages or their cellular products, and that there was no detectable metabolization or conjugation of bilirubin by these cells. In the present study we introduced unconjugated bilirubin into tissue cultures of L-929 fibroblasts, Chang liver cells and fetal rat liver cells to investigate how these mammalian cells would interact with bilirubin.

Materials and methods. Preparation of bilirubin. The preparation of bilirubin and its introduction into tissue culture medium have been described(1).

Tissue culture medium. The medium used for all the experiments consisted of Minimum Essential Medium of Eagle (MEM) (Grand Island Biological Co.) with 10% calf serum (Microbiological Associates), made up in the following manner: 10 ml of calf serum were added to 76 ml of reconstituted MEM. (The MEM was reconstituted by adding 1 ml of Glutamine 200 mM (Microbiological Associates) to 99 ml of MEM.) The 86 ml of this mixture were then added to 14 ml of a bilirubin-albumin solution while stirring slowly. One hundred ml of the final medium contained the required concentration of bilirubin, 3% of human albumin and 10% of calf serum. The pH of this medium was 7.4-7.6 and no gassing with CO_2 was required. The osmolality as determined by the Fiske Osmometer was 370 (or 9.45 atmospheres osmotic pressure). A control medium contained the identical ingredients except for the bilirubin.

L-929 fibroblast culture. Flasks with L-929 fibroblast moistened by a small amount of basal medium (Eagle) with 10% calf serum containing penicillin-streptomycin (100 units per ml) were obtained from Microbiological Associates. The cells were then trypsinized and the medium was replaced by our MEM/calf serum control medium (not containing bilirubin). The cells in suspension were then introduced into Leighton type tissue culture tubes (1 ml of medium containing 150,000 to 200,000 cells per tube). The cultures were incubated at 37°C for 48 hours with the control medium. At this time the medium was replaced with a fresh batch which contained 10 mg% of bilirubin, and tubes were incubated at 37°C for one minute, and 1, 24 and 48 hours respectively. To see whether the results would be different at a temperature at which metabolic activity ceases, replicate cultures were also incubated at 4°C for the same periods. Controls consisted of tubes with medium without bilirubin and of tubes devoid of cells but containing medium with bilirubin. When the cultures were terminated, the supernatant medium was withdrawn and total and direct bilirubin concentrations were measured.

The coverslips were rinsed with saline, fixed in methanol, dried in air and stained with a 2% Giemsa solution in distilled water. The stained coverslips were rinsed with tap water, air-dried, and mounted on glass slides.

Chang liver cell cultures. Leighton tubes containing coverslips with a diffuse monolayer growth of Chang liver cells were obtained from Microbiological Associates. The medium in these tubes was the same as that for the fibroblasts. On arrival in our laboratory the medium was replaced with our MEM-calf serum medium containing 10 mg% of bilirubin and experiments similar to those with the fibroblasts were set up.

Rat liver explants. Gravid Wistar rats in their 18th to 20th day of pregnancy were killed by decapitation. The abdominal cavity was opened under sterile conditions. The fetuses were removed, placed in sterile containers and their abdomens opened. Liver

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TABLE I. Average Percentage Drop of Total Bilirubin Concentration in Supernatant Medium (in Presence and Absence of Cells) After 24 Hr Exposure at 37°C and 4°C.*

Cells	Hr & temp	% Drop of bilirubin	
		With cells	Without cells
L-929 fibroblasts	24 hr, 37°C	20.1	9.2
	24 hr, 4°C	29.3	9.3
Chang liver cells	24 hr, 37°C	29.3	15.5
	24 hr, 4°C	30.3	6.0
Fetal rat liver cells	24 hr, 37°C	37.3	11.7
	24 hr, 4°C	24.3	15.5

* Differences between levels of bilirubin in presence and absence of cells were statistically significant ($p < .001$).

tissue was removed from the fetuses and then cut into tiny fragments. Four fragments of liver measuring about 0.1-0.2 cm in greatest diameter were placed on a coverglass in a Leighton tissue culture tube and covered with 1 ml of our MEM/calf serum control medium. The cultures were incubated at 37°C for 72 hours. At this time the medium was replaced with a fresh batch which contained 10 mg% of bilirubin. The experiments were similar to those with the fibroblasts and Chang liver cells. One experiment was set up with liver tissue obtained from a 3-week-old male rat, and was incubated at 37°C for 24 hours only.

Measurement of bilirubin. Total bilirubin was measured by a modification of the method of Malloy and Evelyn including a modification of the diazo solution as described elsewhere(1). The one minute direct Van den Bergh reaction was used for determination of conjugated bilirubin.

Results. Measurements of bilirubin in supernatant medium. A spontaneous drop of bilirubin concentration in the medium in the absence of cells measured at the end of the 24 hours of incubation ranged from about 9% to about 15% at 37°C and from about 6% to about 15% at 4°C. A sharp increase in the drop of bilirubin in the presence of cells occurred as early as one minute after the introduction of bilirubin into the culture and did not vary significantly for the next 48 hours. This drop in bilirubin concentration ranged from about 20% to 37% at 37°C and from 24% to 30% at 4°C. The results were similar in the 3 cell types tested. The differences between control groups without cells and test groups with cells were statistically significant and were consistent in repeated experiments (P values below 0.001). The data are summarized in Table I.

The values obtained by measuring the 1 minute Van den Bergh reaction in the supernatant medium at both 4°C and 37°C were so small that they fell below the level of sensitivity of our methods. These values ranged from 0-0.4 mg% regardless of the presence or absence of cells in the tissue culture tubes. No conjugation of bilirubin could therefore be detected in our experiments.

Appearance of cells in tissue culture and the effect of bilirubin on their morphology. The L-929 fibroblasts and Chang liver cells were abundant in all experiments and showed no cellular abnormalities. The fetal rat liver cells, however, were only present in small isolated foci and were mixed with other cellular elements such as spindle cells and small round cells. In these latter cultures were many foci of necrotic cells. Cells grown at 4°C up to 48 hours showed no morphological abnormalities.

Ten mg% of bilirubin in the medium did not cause visible damage to the cells. Occasionally some crystals of bilirubin were found outside of cells. This was somewhat more common at 37°C than at 4°C but was not a constant feature. Phagocytosis of bilirubin by the cells was not observed.

Discussion. These results indicated that in tissue cultures of L-929 fibroblasts, Chang liver cells and fetal rat liver cells incubated at 37°C, the drop in concentration of unconjugated bilirubin incorporated into the medium exceeds by far that occurring as a result of spontaneous breakdown. The fall in concentration of bilirubin could be demonstrated as early as one minute after its introduction in the culture medium incubated at both 4°C and 37°C, and did not vary significantly up to 48 hours which was the upper time limit of our experiments. These results were similar to those previously obtained with spleen macrophages from chick embryos (1). They confirmed our original conclusions that the drop of bilirubin in tissue culture

was most probably due to a combination of spontaneous breakdown and adsorption on the cells or cell products and that this constituted a non-specific phenomenon common to various cell types. Conjugation or metabolization could not be demonstrated. While macrophages, L-929 fibroblasts and Chang liver cells are not known to conjugate or metabolize bilirubin, one might have expected conjugation to occur with fetal and 3-week-old rat liver cells(2). The failure to do so *in vitro* may have been due to the scanty growth of the liver cells in tissue culture. On the other hand the drop of total bilirubin with these inadequate cultures of liver cells was equal to that with the heavy cultures of fibroblasts and Chang cells. It appeared, therefore, that the amount of bilirubin adsorbed on the cells was independent of the number of living cells in the culture. It might have actually been adsorbed onto dead cells and cell products as well.

The presence of 10 mg% of bilirubin in the medium did not produce any visible ill effect on the 3 cell types tested—higher concentrations were not tried in this experiment. In the previous study, concentrations of up to 30 mg% of bilirubin failed to affect the macrophages(1).

Summary. The effect of L-929 fibroblasts, Chang liver cells and rat liver cells on 10 mg% unconjugated bilirubin bound to albumin and introduced into tissue culture medium was studied. In the presence of these cells incubated at 4°C or 37°C there was a drop in concentration of bilirubin which exceeded by far that occurring spontaneously. This drop was observed as early as one minute after the introduction of bilirubin in the medium and did not vary appreciably during the next 48 hours. Conjugation or metabolization of bilirubin could not be demonstrated. These results are similar to those previously obtained with chick spleen macrophages and indicate that bilirubin in tissue culture is readily adsorbed on various cells or cell products. The bilirubin had no ill effects on the cells studied.

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Some Effects of Acute Manganese Excess in Rats.* (30351)

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The chronic effects of a high dietary manganese intake have been studied in a number of animals(1-14) and chronic manganese intoxication has been described in man(15-20). However, the effects of an acute manganese load on the organism have not been extensively investigated. To our knowledge only Paterni(21) has reported on this problem.

In the present study rats have been injected subcutaneously with various dose levels of manganese and observations of the effects on a number of physiological parameters made at various time periods.

Materials and methods. Albino Holtzman rats weighing 100 to 550 g were used throughout the study. Observations reported for any specific time period and/or dose levels are compared to controls of equal weight, age and sex. Manganous chloride was diluted in normal saline to give dose levels ranging from 5 to 150 mg of manganese. Controls received an equal volume of normal saline only. At various time periods following subcutaneous administration of manganese, animals were anesthetized with ether and 4 ml blood samples taken from the inferior vena cava. All

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