Bilirubin Toxicity in Human Cultivated Fibroblasts and its Modification by Light Treatment (34724)

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Previous reports have demonstrated the inhibition by bilirubin of cell growth in tissue culture (1, 2). The mechanism thought to be responsible for the inhibitory effects of bilirubin is the uncoupling of oxidative phosphorylation within mitochondria (3). The above studies were carried out in an attempt to explain the relationship of hyperbilirubinemia to central nervous system damage in the neonatal period. In recent years, hyperbilirubinemia has been successfully treated by phototherapy (4, 5), presumably causing the breakdown of bilirubin in the skin by photooxidation (2). In an attempt to correlate these observations, a study was undertaken to determine the effects of bilirubin and light-treated bilirubin upon cultivated human fibroblasts derived from normal individuals.

Materials and Methods. The stock bilirubin solution was prepared immediately prior to use by dissolving 48.6 mg of bilirubin (Pfanstiehl) in 10 ml of 0.1 *M* NaOH and diluting it to 100 ml with 0.1 *M* sodium phosphate buffer, pH 7.4, to give a final concentration of 8×10^{-4} *M* bilirubin. Part of this solution was exposed to fluorescent light for 12 hr (100 W, 1 ft away). The treated and nontreated bilirubin solutions were diluted 1:16 in the culture medium producing a final concentration of 5×10^{-5} *M* bilirubin.

Human skin biopsies were obtained from normal subjects using methods previously described (6). The skin samples were cut into several pieces, immobilized under cover slips in 35-mm plastic petri dishes (Falcon) containing culture medium (nutrient mixture F10, supplemented with 15% fetal calf serum, and 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone) and placed in a 5% carbon dioxide atmosphere at 37°. After 3–4 weeks the cells were subcultured and maintained in culture until an adequate population of cells was available for study. Replicate aliquots of cells suspensions, usually 3×10^6 cells, were planted and fed daily with medium (6). After 4 days the medium was replaced by (i) medium, (ii) medium containing bilirubin, and (iii) medium containing light-treated bilirubin. Cells were harvested at 0, 6, 24, and 48 hr for cell count, protein determination, and enzyme analysis.

The cells were rinsed twice with normal saline. Four ml of EDTA (1:5000)–trypsin (0.25%) solution were added, and the resulting cell suspension was centrifuged at 750*g* for 10 min. The cell pellet was resuspended in 2 ml of 0.3 *M* sucrose, pH 7.4, and frozen and thawed three times. A sample of the cell homogenate was used for enzyme analysis and the remainder was centrifuged at 4° in an IEC, HR-1 Head at 350*g* for 10 min to remove nuclear and cellular debris. The supernatant fraction was subjected to centrifugation at 4300*g* for 10 minutes, and the microsomal fraction by centrifugation at 78,484*g* for 37 min. The remaining supernatant fraction was defined as the high speed supernatant.

The following enzyme analyses were performed: Acid phosphatase activity was determined at pH 4.90 by using *p*-nitrophenol phosphate as substrate (7); α-glucosidase activity was determined by the method outlined by Nitowsky and Grunfeld (8); glucose-6-phosphate dehydrogenase activity was determined by the method of Glock and McLean using glucose-6-phosphate and 6-

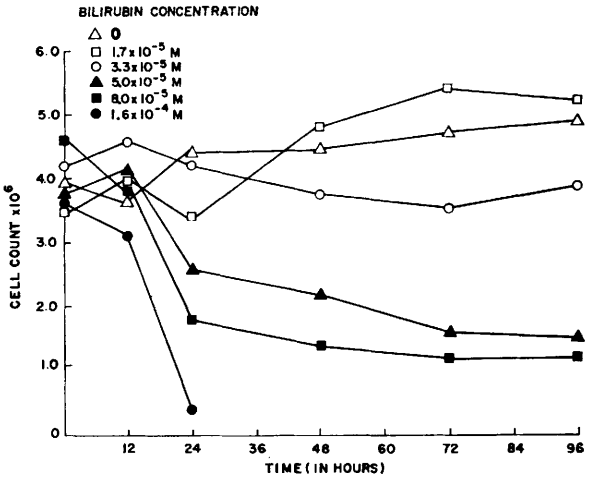
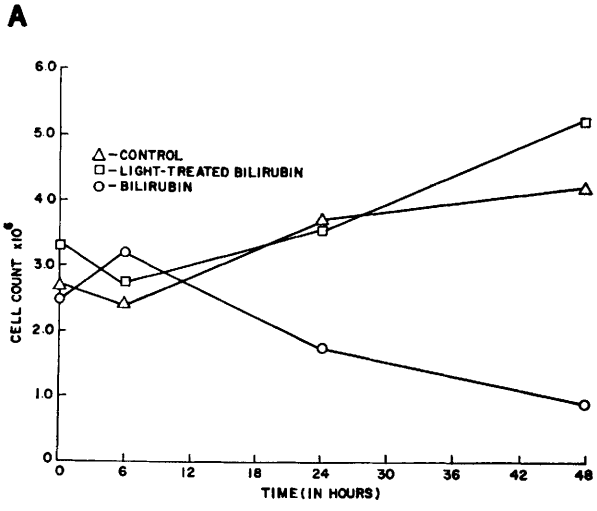


FIG. 1. The relationship of bilirubin concentration to cell growth.

phosphogluconate as substrates and measuring the reduction of NADP at 340 mμ (9); lactate dehydrogenase was determined by the method of Wroblewski and LaDue (10); cytochrome *c* oxidase activity was determined by reduction of cytochrome *c* (Sigma) with sodium hydrosulfite and monitoring its reoxidation at 550 mμ (11); succinic-cytochrome *c* reductase activity was determined by preincubating 0.1 ml of sample in a reaction mixture of 1.2% bovine serum albumin, 6×10^{-3} *M* KCN, 1.2×10^{-2} *M* succinate and 0.1 phosphate buffer, pH 7.4, after which 9.3 m*M* cytochrome *c* was added and the reaction monitored at 550 mμ (12); and NADH-cytochrome *c* reductase activity was determined using the same method with the succinate replaced by 2.7×10^{-4} *M* NADH. Protein was determined by the method of Lowry *et al.* (13) and cell counts were performed utilizing a model F Coulter counter.

Results. Bilirubin in concentrations greater than 3.3×10^{-5} *M* caused inhibition of cell growth (Fig. 1). The inhibitory effect of 16×10^{-5} *M* bilirubin was not observed when a similar concentration of light-treated bilirubin was used (Fig. 2).

Bilirubin (5×10^{-5} *M*) or light-treated bilirubin (5×10^{-5} *M*) had no demonstrable effect upon the specific activities of acid



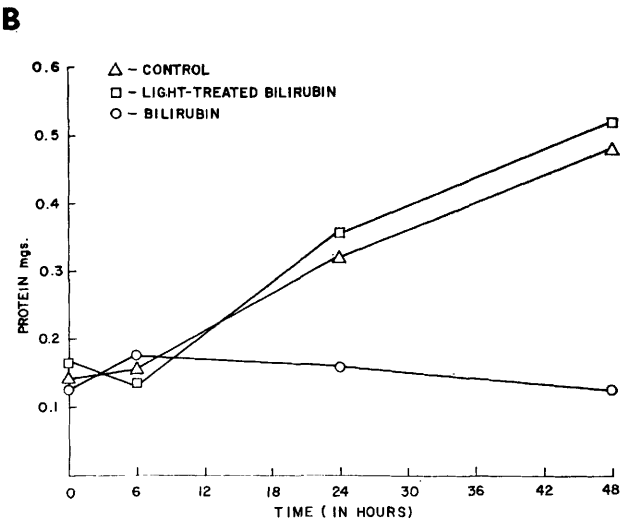


FIG. 2. The effect of bilirubin and light-treated bilirubin upon cell number (A); and total protein (B.)

phosphatase, α-glucosidase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, cytochrome *c* oxidase, or succinate-cytochrome *c* reductase. On the other hand, the specific activity of NADH-cytochrome *c* reductase was reduced by about 35% in the cultures containing 5×10^{-5} *M* bilirubin compared to either control cultures or cultures containing light-treated bilirubin. In Table I is shown the inhibition of NADH-cytochrome *c* reductase when cells are grown in the presence of different bilirubin concentrations. These experiments were carried out with duplicate samples and were repeated three times. In each instance, similar findings were observed. In Table II are results of varying bilirubin concentrations added directly to the assay system for NADH-cytochrome *c* reductase. Bilirubin added directly to the assay system inhibits NADH-cytochrome *c* reductase activity to a degree comparable to that observed when bilirubin is added to cell cultures. Light-treated bilirubin had no inhibiting effect upon the enzyme activity when added directly to the assay mixture.

TABLE I. Effects of Bilirubin upon NADH-Cytochrome *c* Reductase Activity[a] in Cell Cultures.

Bilirubin conc (*M*)	NADH-Cytochrome *c* reductase activity	
	Cells + bilirubin	Cells + light-treated bilirubin
0	0.72	0.69
1×10^{-5}	0.74	0.71
2×10^{-5}	0.68	0.74
3×10^{-5}	0.62	0.64
5×10^{-5}	0.48	0.68
10×10^{-5}	0.12	0.70
15×10^{-5}	0.01	0.69

[a] μmoles of cytochrome *c* reduced/min/mg of protein.

TABLE II. Effect of Bilirubin upon NADH Cytochrome *c* Reductase Activity.[a]

Bilirubin conc	Bilirubin	Light-treated bilirubin
0	0.75	0.75
3×10^{-5}	0.68	0.79
5×10^{-5}	0.46	0.71
10×10^{-5}	0.20	0.68
15×10^{-5}	0.06	0.75

[a] μmoles of cytochrome *c* reduced/min/mg of total protein.

Discussion. These observations utilizing normal human diploid cells confirm the previously reported toxicity of bilirubin in tissue cultures (1, 2). In contrast to the findings of Cowger *et al.* (1) using L-929 cells, bilirubin toxicity was demonstrated in the presence of albumin in our culture medium. This difference might be explained by differ-

ence in cell types or by Ernster's observation (3) that bilirubin toxicity in the presence of albumin is related to the molar ratio of bilirubin to albumin. In our experiments the bilirubin:albumin ratio is 100:1, exceeding the 1:1 ratio required to produce bilirubin toxicity (3). The demonstration of inhibition of NADH-cytochrome *c* reductase by bilirubin in a purified enzyme system (1), in human fibroblast cultures and in the *in vitro* enzyme assay suggest that bilirubin toxicity *in vivo* may be due to either the uncoupling of oxidative phosphorylation or a block in intracellular respiration. The observation of similar degrees of inhibition of NADH-cytochrome *c* reductase activity when bilirubin is added during growth or directly to the assay system suggests inclusion of bilirubin within the mitochondria and consequent inhibition of the assay system.

The toxic effect of bilirubin upon cell growth and its inhibition of NADH-cytochrome *c* reductase were prevented when bilirubin was exposed to light. The mechanism by which bilirubin is rendered "nontoxic" is thought to be the splitting of its tetrapyrrole structure into dipyrroles (2). Whatever the exact mechanism by which light renders bilirubin "nontoxic," the products produced do not appear to have adverse effects upon either cell growth or enzyme activity.

Summary. The effect of bilirubin and light-treated bilirubin upon cultivated human fibroblasts was studied. The toxic effects of bilirubin upon cell growth and inhibition of NADH-cytochrome *c* reductase activity which were demonstrated could be prevented when bilirubin was exposed to light. The products of light-treated bilirubin did not produce adverse effects upon cell growth or enzyme activity.

We thank Greta Spells and Marilyn Swae for excellent technical assistance. These studies are supported by grants from the National Institute of Health HD 04252 and HD 00036.

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Received Oct. 29, 1969. P.S.E.B.M., 1970, Vol. 134.