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Bilirubin Toxicity in a Neuroblastoma Cell Line N-115: I. Effects on Na^+K^+ ATPase, [^{3}H]-Thymidine Uptake, L-[^{35}S]-Methionine Incorporation, and Mitochondrial Function

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ABSTRACT. Though bilirubin is reported to affect a variety of cellular functions, the primary target of its toxic effect is still not known. A major problem in understanding this is the wide variation in results reported by different groups. This is probably due to the differences in stability of bilirubin solutions arising from large differences in bilirubin:albumin molar ratios used in experiments. Hence in studying the toxic effects of bilirubin in tissue culture systems, it is important to be certain that the bilirubin is maintained in solution throughout the time of the exposure to bilirubin. Spectrophotometric measurements have shown that bilirubin is stable in Dulbecco's modified Eagle medium solution at bilirubin:albumin molar ratios up to 3. Under these defined conditions, bilirubin was found to affect Na^+K^+ ATPase, [^{3}H]-thymidine uptake, L-[^{35}S]methionine incorporation into protein and mitochondrial function at bilirubin concentrations up to 125 μM and bilirubin:albumin molar ratio of 1.5. Toxic effects on all parameters measured were evident at bilirubin:albumin molar ratio of 1.5 after a minimum of 2 h of exposure. No effect was evident at a bilirubin:albumin molar ratio below 1. Although it is not possible to identify with certainty the primary target, the effect on mitochondrial function appeared earlier and was more profound than that seen with the other assessed functions. (*Pediatr Res* 25:364–368, 1989)

Abbreviations

B/A, bilirubin:albumin molar ratios
HSA, human serum albumin
MTT, 3-(4,5 dimethylthiazol-y-yli),-2,5-diphenyl tetrazolium bromide
DMEM, Dulbecco's modified Eagle medium

The mechanism of bilirubin encephalopathy has been studied extensively over the past decade. Data has been obtained from tissue examinations using light and electron microscopy (1, 2), *in vitro* assessment of bilirubin toxicity in different neural and nonneural tissues (3) and, more recently, *in vivo* studies of different brain cell functions in Gunn rats suffering from hereditary bilirubin encephalopathy (4).

The major biochemical defect underlying bilirubin encephalopathy has yet to be determined. Studies conducted on neural tissue demonstrated that bilirubin can impair a large number of cellular functions. Among them are: changes in energy metabolism (4–6), impairment of various membrane functions and intracellular key enzymes—such as Na^+K^+ ATPase, glutamate decarboxylase, lactate dehydrogenase, protein kinase, to name a few (7–11)—alterations in the physical and functional state of the cell membranes (12–16), inhibition of both DNA (17, 18) and protein synthesis (19–22), changes in carbohydrate metabolism (23, 24), and modulation of neurotransmitter synthesis (25). However, variation in different cellular functions, the use of concentrations of bilirubin higher than usually encountered in clinical situations, and the use of varying albumin concentrations, thus altering the B/A, may account for the multiplicity of effects and inconclusive results. The use of bilirubin without added albumin, or the use of high bilirubin concentrations at high B/A causes rapid aggregation of bilirubin (26–29). Once aggregates are formed, changes in free bilirubin concentration occur, and hence may give rise to variable toxicity as the free form of bilirubin seems to be the reactive form.

As bilirubin can interfere with a number of cellular functions, it is clearly important to establish which functions are damaged first in particular cell populations. In the present study, using stable bilirubin-HSA mixtures at B/A of 0.8 and 1.5, the effect of bilirubin on $^{42}K^+$ influx, [^{3}H]thymidine uptake, L-[^{35}S]methionine uptake, and mitochondrial function in the neuroblastoma cell line N-115 in culture is investigated.

MATERIALS AND METHODS

Chemicals. All reagents are analytic grade chemicals and include bilirubin (Lot #13F0846), HSA (fraction v, essentially FFA free), MTT obtained from Sigma Chemical Co. (St. Louis, MO), DMEM PBS, and FCS obtained from Grand Island Biological Co. [^{3}H]thymidine (sp act, 15.1 Ci/mmol) and L-[^{35}S]methionine (sp act 1129 Ci/mmol) were purchased from Dupont, Mississauga, Ontario, Canada. $^{42}K^+$ was produced by irradiation of K_2CO_3 at the Slow Poke reactor, University of Alberta. The sp act was 0.36 mCi/mmol at the end of the radiation, and the experiment was carried out within 4 h.

Preparation and stability of bilirubin-HSA mixtures. A stock solution of HSA (mol wt, 68 000) was prepared as a 50-μM solution in 50-mM Tris buffer (pH 7.4) and sterilized by filtration. Immediately before the experiments, a stock solution of 10-mM bilirubin in 0.1-N NaOH was prepared. The composition was verified by high pressure liquid chromatography to contain

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92% of the IXα moiety, the rest being XIIIα and IIIα. No other bile pigments were detected. Thus the bilirubin preparation was used without further purification. Then 5.85 mg bilirubin was dissolved in 0.5 mL 0.1-N NaOH. Once in solution, 0.5 mL of diluent at pH 7.8 containing 0.45% NaCl and 0.45% Na_2CO_3 was added. The bilirubin and albumin solutions were mixed in various vol to achieve the desired B/A.

MTT was made up as a 5 mg/mL solution in PBS (pH 7.4) and was filtered to sterilize. This yellow solution was stable for several wk when stored in the dark at 4°C. Just before use, one part MTT was mixed with nine parts of protein-free medium.

To determine the stability of bilirubin-HSA mixtures in 50-mM Tris buffer (pH 7.4), the freshly prepared stock bilirubin solution was centrifuged for 5 min at 10 000 × *g* to remove the undissolved material. The stability of bilirubin-HSA mixtures, using 5-μM HSA and bilirubin concentrations ranging from 4.5–36 μM, giving B/A from 0.9–7.2, was measured by spectrophotometry (28). The mixtures of different B/A were made up in 6-cm tissue Petri culture dishes and diluted to a final vol of 10 mL with Tris buffer, and incubated at 37°C in the dark. At different time intervals (0, 1, 2, and 24 h), 1 mL of solution was removed into an Eppendorf centrifuge tube, centrifuged at 10 000 × *g* for 5 min, and absorbance at 460 nm measured. All the experiments were carried out in a dimly lit room to avoid bilirubin photodegradation. As DMEM contains amino acids, minerals, and vitamins, bilirubin stability may differ in the medium than in Tris buffer. Hence these experiments were repeated with DMEM. (DMEM also contains a dye, methyl red, which has an absorption maximum of 550 nm at pH 7.4. Interference of bilirubin absorption in culture medium was compensated for by using DMEM as the blank when measurements were made.)

N-115 cells. N-115 cells were seeded on 35-mm culture dishes and allowed to grow in standard DMEM and 10% FCS at 37°C in a 5% CO_2 humidified atmosphere for 12 h. Then the medium was removed by suction and replaced with 1 mL protein-free media (30) plus HSA for another 12 h, before the experiment with bilirubin was set up. Bilirubin was prepared as described before and added to the experimental medium to make final bilirubin concentrations of 75, 100, or 125 μM and B/A of 0.8 and 1.5. The addition of bilirubin was immediately followed by an amount of 0.1-N HCl equivalent to the amount of NaOH to restore the pH to 7.4. Control cells were seeded and grown as above with HSA added. NaOH (0.1 N) and 0.1-N HCl were added to the media, with no bilirubin, in the same vol as in the bilirubin-treated cells.

$^{42}K^{+}$ influx. To assess the effect of bilirubin on Na^+ K^+ ATPase activity, the following series of experiments were carried out. Cells were seeded at a density of 4–5 × 10^5/plate and prepared as described above. Bilirubin in concentrations of 75 μM and 100 μM and B/A 0.8 and 1.5 was added to the test cells and incubated for 2 and 4 h. To determine whether the bilirubin effect, if any, is on passive or active transport of K^+, another set of similar experiments was done in which the cells were incubated in media containing 0.5-mM ouabain for 10 min before the addition of $^{42}K^+$. At 1 h before the end of the incubation period, 2-mM $^{42}K^+$ was added. At the end of the 60-min incubation period, the culture dishes were placed on ice, the medium was removed, and the cells were washed five times with ice-cold PBS (31). Then the cells were harvested in 0.5 mL PBS, scraped off into an Eppendorf test tube, and counted in a Beckman Gamma Counter (Beckman Instruments, Fullerton, CA) for 1 min. The background counts were always less than 1% of the total and were subtracted from the total counts.

$^{42}K^+$ influx and [^{3}H]thymidine uptake. To compare the effect of bilirubin on $^{42}K^+$ influx and [^{3}H]thymidine uptake, the cells were grown as before and exposed to 125-μM bilirubin with B/A of 0.8 and 1.5 for 2, 4, and 6 h. At 1 h before the end of the incubation period, the cells were pulse labeled with $^{42}K^+$ and handled as described above. At the time of the addition of $^{42}K^+$, cells were also pulse labeled with [^{3}H]thymidine, 2 μCi/dish for 60 min to assess thymidine uptake by the cells. Uptake was assessed as reported previously (18).

MTT assay. The MTT assay has been used effectively for assessment of cell viability (32). It assesses the ability of the mitochondria to cleave the dye to form a dark blue formazan. To achieve this, the cells were seeded and grown as described above and exposed to 100-μM bilirubin, B/A ratio of 1.5, for 0, 1, 2, 3, and 4 h. MTT was prepared and sterilized as described before (18, 32). At 60 min before the end of the exposure to bilirubin, 100 μl of the MTT was added. At the end of the incubation period, the cleaved dye, seen as blue crystals within the cells, was dissolved in 1 mL of isopropanol-HCl (0.04 N) by agitation with repeated pipetting until a blue solution was obtained. The absorbance of the individual cultures was then read in a diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) with a test wavelength of 570 nm and a reference wavelength of 630 nm. The difference in absorbance is a direct measure of mitochondrial function and cell viability (32).

L-[^{35}S]methionine incorporation into protein. N-115 cells were seeded and grown as before and exposed to 100-μM bilirubin, at B/A ratio of 1.5, for 0, 1, 2, 3, and 4 h. At 1 h before the end of the incubation period, the cells were pulse labeled with 10 μL of 1/100 dilution of a stock solution of L-[^{35}S]methionine for 60 min. At the end of the incubation, the medium was removed into an Eppendorf test tube, the cells were suspended in 0.5 mL PBS and scraped off into another Eppendorf test tube. Proteins in the medium and in the cells were precipitated with 1 mL of 10% trichloroacetic acid solution. After centrifugation at 10 000 × g for 5 min, the supernatant was removed and the pellet redissolved in 1-N NaOH. Half of the pellet was measured for radioactivity by liquid scintillation counting using ACS (Amersham Corp., Arlington Heights, IL) as scintillant and the other half used for protein estimation (33).

RESULTS

Bilirubin stability. Figure 1 demonstrates the stability of 35-μM bilirubin in 50-mM Tris buffer solution at different B/A in the range of 0.5 to 8 at 37°C. In the albumin-free state, virtually all of the bilirubin precipitated out of the solution immediately. At a B/A of 1 or less, the absorbance of the bilirubin solution remained unchanged over a 24-h period. The bilirubin solution became less stable as the B/A increased and declined to 60% of the initial level at a B/A of 8. The same pattern was seen when DMEM was used instead of Tris buffer.

$^{42}K^+$ influx. The effect of bilirubin on $^{42}K^+$ influx is given in Table 1. The total $^{42}K^+$ influx is inhibited by bilirubin only at a concentration of 100 μM and B/A 1.5, and this becomes manifest only after 4 h of exposure. In this time frame, the portion affected is only the ouabain inhibitable or active (Na^+K^+ATPase) com-

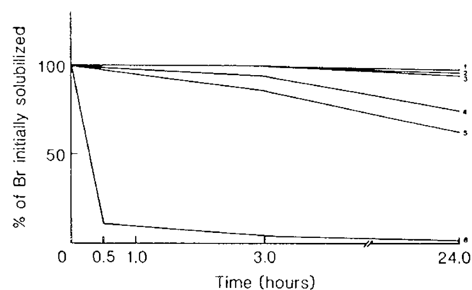

Fig. 1. The solubility of 35-μM bilirubin (*Br*) in 50-mM Tris buffer at B/A of 0.5 (*1*), 1.0 (*2*), 2.0 (*3*), 4.0 (*4*) and 8.0 (*5*). Curve (*6*) represents 35-μM bilirubin in Tris Buffer in the absence of albumin. Absorbences were measured at 0, 0.5, 1, 3, and 24 h. In the absence of albumin, bilirubin precipitates out of solution within 30 min, whereas at B/A up to 3, up to 10% bilirubin is lost by 24 h. Similar results were obtained for DMEM.

ponent. No effect was seen on the ouabain resistant, or passive influx component.

$^{42}K^+$ influx and [^{3}H]thymidine uptake. The effect of exposure to 125-μM bilirubin, at B/A of 1.5, on $^{42}K^+$ influx and [^{3}H] thymidine uptake, is depicted in Figure 2. Uptake of [^{3}H]thymidine was decreased by 40% of control values within 4 h of exposure. $^{42}K^+$ influx was affected only after 4 h of exposure, and to a lesser degree than the effect on [^{3}H]thymidine uptake.

MTT assay. The mitochondrial function measured as the difference in absorbance at 560 and 630 nm in the MTT assay is given in Table 2. The results show a decrease in the ability of the bilirubin-treated cells to cleave the MTT dye shortly after the exposure. Although a 43% reduction in activity was seen after 2 h of exposure to bilirubin, the most pronounced effect on viability (63% reduction of activity) was seen after 4 h of exposure.

L-[^{35}S]methionine incorporation into protein. At the end of 2, 3, and 4, h exposure of the cells to 100-μM bilirubin, B/A of 1.5, there was a significant decrease in L-[^{35}S]methionine incorporation into protein compared to control (Fig. 3). The decrease in incorporation into protein was noticed in proteins extracted from cells and media.

Table 1. Effect of bilirubin treatment of N-115 cells on *$^{42}K^+$ influx**

B/A	Bilirubin concentration (μM)	$^{42}K^+$ influx—cpm/μg DNA/h (2 hr bilirubin exposure) Ouabain resistant	Ouabain inhibitable	$^{42}K^+$ influx—cpm/μg DNA/h (4 hr bilirubin exposure) Ouabain resistant	Ouabain inhibitable
	Control	38 ± 3	210 ± 4.3	45 ± 6	202 ± 2.5
0.8	75	36 ± 5	207 ± 4.5	44.9 ± 3	208 ± 4.7
	Control	44 ± 3	214 ± 5.4	52 ± 0.5	207 ± 6.9
0.8	100	42 ± 5	208 ± 5.7	40 ± 0.3	207 ± 3.5
	Control	37 ± 2	166 ± 4.8	44 ± 4	166 ± 3.4
1.5	75	31 ± 6	167 ± 3.5	40 ± 4	123 ± 4.6†
	Control	41 ± 4	169 ± 3.1	42 ± 3	163 ± 4.5
1.5	100	36 ± 5	160 ± 3.0	37 ± 3	130 ± 1.3‡

* Values given are mean ± SD from three measurements × 2.
† $p = 0.00001$.
‡ $p = 0.0003$.

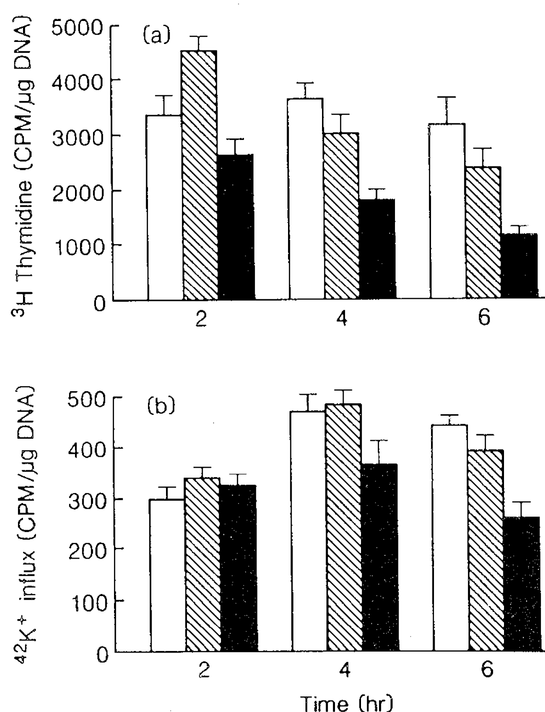

Fig. 2. The effect of bilirubin (125 μM) on [^{3}H]thymidine uptake (*a*) and $^{42}K^+$ influx (*b*) by N-115 cells. Data is expressed as CPM/μg DNA accumulated over a 1-h period. □, control; ▧, B/A 0.8; ■ B/A 1.5.

Table 2. *MTT assay for viability of control and bilirubin-treated cells**

Duration of exposure (h)	Difference in absorbance (A560–A630) Control cells	Bilirubin-treated cells	% Control
0	0.2548 ± 0.0122	0.2502 ± 0.0045†	100
1	0.2318 ± 0.0062	0.2071 ± 0.0096	87
2	0.222 ± 0.0172	0.1203 ± 0.0072	57
3	0.2066 ± 0.0046	0.1203 ± 0.0072	60
4	0.2153 ± 0.0114	0.0799 ± 0.0133	37

* Cells were treated with 100-μM bilirubin, B/A 1.5. An equivalent amount of HSA was added to control cells.
† Mean ± SD of two triplicates analyses.

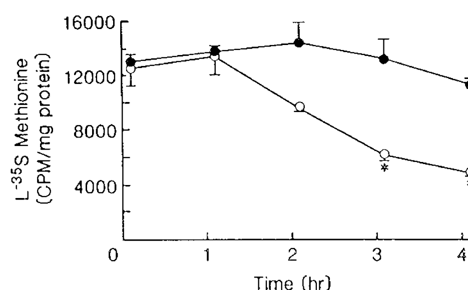

Fig. 3. The effect of 100-μM bilirubin on L-[^{35}S]methionine uptake by N-115 cells at B/A 1.5 (○) compared to controls (●). Data is expressed as cpm mg protein accumulated over a 1-h period.

DISCUSSION

The mechanism of bilirubin toxicity to the central nervous system has been debated extensively over the past years. The difficulties in analyzing the results and the inability to point to a primary bilirubin target stems from variation in experimental designs, the use of different animal models and difficulties in correlating the chemical, biochemical, and clinical knowledge of the bilirubin molecule in *in vitro* and *in vivo* experiments.

Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, the hyperbilirubinemic Gunn rat served as the model; in the other, brain cells from normally developed animals were used. The difference between the two is a major one. The use of the Gunn rat as a model for bilirubin encephalopathy is based on the assumption that the damage seen is primarily due to bilirubin. Studies in Gunn rats have shown that bilirubin is indeed toxic to the mitochondria (4), causes changes in membrane morphology (2), affects glycolytic (23, 24) and other cellular enzymes (9), modulates neurotransmitter synthesis (25) and may inhibit protein (19–22) and DNA (17) synthesis. Though extensive damage to the nervous system in the Gunn rat can be attributed to bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded (34, 35).

Exposure of neural cells to bilirubin for a limited time period may not properly reflect the more prolonged influx of bilirubin encountered in the clinical situation. Yet the input from many studies points to damaging effects. Bilirubin was shown to impair mitochondrial reactions (5, 6, 18), and to inhibit various cellular enzymes either directly (7, 8, 10, 11) or via alterations in the membranes (12–16). Despite this, no specific target has been singled out as the primary one for bilirubin toxicity.

A major concern when experimenting with a B/A that exceeds

one, is the instability of the bilirubin solution leading to formation of bilirubin aggregates and coaggregates of bilirubin and albumin (26–29). Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been addressed in experiments dealing with bilirubin toxicity *in vitro* (24, 37–41). In previously reported data, when either free bilirubin or bilirubin in excess of albumin was used, the toxic effect appears to be an instant one, within minutes of exposure (4–7, 10, 15). One often used solution to the bilirubin stability problem is raising the pH of the buffer to 8.2 or higher (16, 42, 43). This proves to be an impractical approach when experimenting with live cells. When complexed with HSA, bilirubin in solution is stable at different concentrations (27). In carrying out the studies herein described, attention is given to the stability of bilirubin in DMEM solutions. Under these conditions, where bilirubin is maintained in solution, toxicity is slower in occurring and is dependent on both the amount of free bilirubin and the time of exposure. Direct interaction of bilirubin with the purified enzymes, as opposed to interaction with the whole cell, may well be the reason for the time difference, but one cannot exclude the possibility that toxicity was delayed or did not occur as a result of bilirubin instability in solution.

In the present investigation, we have shown that bilirubin, at concentrations of 35–125 μM and B/A of 1.5, is stable over a 24-h period in the medium (DMEM) for the neuroblastoma cell line N-115. When applying this approach to cell studies, it was noticed that at 50 μM bilirubin and B/A $\leq$ 0.8, bilirubin binding by the N-115 cell in monolayer culture was negligible. However, at B/A 1.5, where loss of bilirubin after 24 h was less than 10%, cellular uptake of bilirubin in 2 h was found to be 110 ng/μg DNA (18).

In the studies herein reported, the effect of bilirubin on four vital cellular functions—mitochondrial activity, protein synthesis, DNA synthesis, and ion transport—is evident. A significant reduction in mitochondrial activity is seen within 2 h of exposure. This effect seems to occur early and is more pronounced than the effect seen on [^{3}H]thymidine uptake, L-[^{35}S]methionine incorporation into protein or $^{42}K^+$ influx. The bilirubin effect on $^{42}K^+$ influx is ouabain sensitive, and is a reflection of the effect of bilirubin on Na^+K^+ ATPase activity. In all these instances, the effects seem to be dependent on the B/A, bilirubin concentration, and the duration of exposure. From these studies, it is not possible to single out conclusively the primary target for bilirubin toxicity, although the data suggest an earlier and more pronounced effect occurring with mitochondrial function. The effect on L-[^{35}S]methionine incorporation into protein seems to develop later in the course of the bilirubin exposure and is less pronounced then that seen with [^{3}H]thymidine uptake. It is difficult from these experiments to determine whether these two observations are a direct result of bilirubin toxicity or secondary effects arising from the initial effect of bilirubin toxicity on mitochondrial function.

It is therefore concluded that in studies involving cells in culture, where it becomes important to know that the amount of bilirubin utilized remains stable and in solution, the optimal B/A to use is <3. In this fashion, reproducibility of conditions related to bilirubin toxicity of the cells can be achieved. Using this approach, it has been demonstrated that bilirubin affects mitochondrial function, [^{3}H]thymidine uptake, L-[^{35}S]methionine incorporation into protein and Na^+-K^+ ATPase activity of the N-115 cell. As mitochondrial dysfunction precedes the other three effects and as ATP is required for protein and DNA synthesis, as well as for K^+ transport, these results point to the possibility that mitochondria may be the primary target of bilirubin toxicity.

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Erratum

There was an error in Table 3 of the article by J. Ramet, et al. titled "Effect of Maturation on Heart Rate Response to Ocular Compression Test during Rapid Eye Movement Sleep of Human Infants" (Pediatr Res 24:477–480, 1988). The headings of columns 1 and 2 were transposed. **The correct Table 3 should read:**

Table 3. *Results of stepwise regression for general measured variables**

Independent variables	Dependent variables	Partial coefficients	Partial R^2	*p*
GA	Longest RR	55.3	0.35	<0.0001
PNA		10.2	0.30	<0.0001
GA	%RR maximum	114	0.38	<0.001
PNA		2.4	0.31	<0.001
GA	Latency	136	0.28	<0.002
PNA		25.8	0.23	<0.001

* Variables were significantly influenced by PNA and GA.