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Reassessment of the Unbound Concentrations of Unconjugated Bilirubin in Relation to Neurotoxicity *In Vitro*

J. DONALD OSTROW, LORELLA PASCOLO, AND CLAUDIO TIRIBELLI

Research Service (151L), VA Puget Sound Health Care System, Seattle, Washington 98108-1597, U.S.A., and the GI/Hepatology Division, Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195, U.S.A. [J.D.O.]; and the Liver Study Center, University of Trieste, I-34012 Trieste, Italy [L.P., C.T.]

ABSTRACT

Most studies of the cellular toxicity of unconjugated bilirubin (UCB) have been performed at concentrations of unbound UCB (B_F) that exceed those in the plasma of neonates with bilirubin encephalopathy. We assessed whether UCB could be toxic to neurons and astrocytes at clinically relevant B_F values ($\leq$1.0 μM), a range in which spontaneous precipitation of UCB would be unlikely to occur, even though B_F exceeded the aqueous saturation limit of 70 nM. A meta-analysis yielded twelve published studies that had determined the *in vitro* effects of UCB on the function of cultured neurons or astrocytes at calculable B_F values $\leq$ 1.0 μM. B_F values were recalculated from the stated UCB, albumin, and chloride concentrations by applying affinity constants derived from ultrafiltration of comparable solutions containing ^{14}C-UCB and delipidated human serum albumin. At B_F slightly above aqueous solubility, UCB impaired mitochondrial function and viability of astrocytes. Exposure of neuroblastoma and embryonic neuronal cell lines to B_F above 250 nM impaired cellular proliferation and mitochondrial function and increased apoptosis. Purified UCB inhibited the uptake of glutamate into astrocytes at B_F as low as 309 nM and induced apoptosis in brain neurons at B_F as low as 85 nM. UCB can impair various cellular functions of astrocytes and neurons exposed to B_F near or modestly above its aqueous solubility limit, at which UCB exists as soluble oligomers and metastable microaggregates. The results render doubtful the long-held concept that precipitation of UCB in or on cells is required to produce neurotoxicity. **(*Pediatr Res* 54: 98–104, 2003)**

Abbreviations

UCB, unconjugated bilirubin
B_F, concentration of free (unbound) UCB
B_T, total UCB concentration
HSA, human serum albumin
K_F, corrected affinity constant of HSA for UCB
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM, Dulbecco's Minimal Essential Medium
LDH, lactate dehydrogenase
T_3, triiodothyronine
DAPI, 4′, 6-diamidino-2-phenylindole

The moderate "physiologic" jaundice that develops after birth may be neuroprotective for the neonate (1), owing to the potent antioxidant properties of UCB (2, 3). By contrast, if the underlying immaturity of the hepatic transport processes or the postnatal increases in production and enterohepatic circulation of UCB are more severe (4), marked neonatal jaundice occurs, which may result in reversible neurotoxicity (bilirubin encephalopathy) (5, 6). This may progress to precipitation of UCB in focal areas of the CNS with permanent neurologic damage (kernicterus) (5).

The UCB that enters the CNS is derived from the free fraction of plasma UCB (B_F) that is not bound to plasma proteins and lipoproteins (6). B_F levels in plasma are normally very low, as a result of the tight binding of UCB to two sites on HSA (7). Recent data indicate that the affinity for UCB decreases markedly as HSA concentration increases (8, 9) and when chloride is added (9). Therefore, the accepted affinity constant of 6×10^7 L/mol (10), determined at an HSA concentration of 60 μM, overestimates by an order of magnitude the true affinity constant at physiologic albumin and chloride concentrations (9), with consequent marked underestimation of B_F. In addition, most published studies of the neurotoxicity of UCB have been performed at total UCB levels vastly higher than those seen in jaundiced neonates with

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Correspondence: Jay Donald Ostrow, M.D., Research Service (151L), VA Puget Sound Health Care System, Seattle Division, 1660 South Columbian Way, Seattle WA 19108-1597, U.S.A.; e-mail: jdostrow@medicine.washington.edu
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reversible bilirubin encephalopathy, and thus have questionable relevance to the clinical manifestations of neurotoxicity (11, 12).

In the present work, we have applied the affinity constants of UCB for HSA (K_F), derived by serial ultrafiltration (9), to recalculate the B_F levels present in published *in vitro* studies of UCB toxicity to neurons and astrocytes. Our aim was to test the hypothesis that *in vitro* neurotoxicity of UCB could be observed at B_F values of 1.0 μM or less, in the range at which spontaneous precipitation of UCB would be unlikely to occur, even if B_F was above the aqueous saturation limit of 70 nM. In 12 studies performed at relevantly low total UCB concentrations, toxicity usually occurred at B_F levels near or modestly above the aqueous solubility of UCB.

METHODS

Selection of papers for meta-analysis. We searched PubMed for papers under the following headings: bilirubin + (cells, cultured or cell lines) + (astrocytes or neurons). After eliminating duplicates and adding two related papers by Silberberg *et al.* (12a,b), we had 28 references. Of these, all but 12 were eliminated for the following reasons: paper published in Chinese 1; review article without original data, 1; did not perform *in vitro* incubations with UCB, 5; data duplicated results in a paper by the same group that was selected for meta-analysis, 1; only uptake and binding of UCB were studied, not toxicity, 1; incubations included whole human or bovine serum, or BSA, precluding estimation of B_F values by applying our ultrafiltration-derived K_F values for binding of UCB to delipidated HSA, 4 (also, in two of these, the source and purity of UCB were not given, one studied only UCB photodegradation products, and one performed studies only at B_F values of ≥ 5 μM); studies were done only at B_F values of 5 μM or greater, 2; studies were done at B_F values below 5 μM, but examined only recovery of function after bilirubin washout and the control data duplicated results in another paper by the same group, 1.

One of the 12 papers selected one was done only at a single B_F value of 0.5 μM in the absence of albumin (13), and in part duplicated data from another paper by the same group (14) that was performed over a range of B_F values. In another selected paper (15), studies were done at B_F values below 5 μM, but no toxicity was observed at B_F values ≤ 1 μM. In three of the papers selected (13, 14, 16), the stock UCB solution was markedly supersaturated, so that precipitation and degradation likely occurred; thus, the true threshold for UCB toxicity may have been lower than the calculated B_F values. Another selected paper studied B_F values below 5 μM, but there were no studies done at B_F values between 383 and 1761 nM, so that the true threshold could not be evaluated (17).

Calculations. B_F levels were calculated from a model that assumes independent binding of UCB to two sites on albumin, using equation 1 (10), where k_1 and k_2 are the binding constants for the first and second sites, respectively:

$$\frac{B_T - B_F}{[HSA]} = \frac{B_F \times k_1}{1 + (B_F \times k_1)} + \frac{B_F \times k_2}{1 + (B_F \times k_2)} \qquad (1)$$

Applying the Solver function of Microsoft Excel 6.0 (Microsoft Corp, Redmond, WA, U.S.A.) to equation 1, B_F values were calculated from the total UCB (B_T) and total albumin [HSA] concentrations given in the selected papers, using affinity constants of solutions containing comparable concentrations of delipidated HSA and chloride. The value for k_1 was set equal to the first site affinity constant (K_F) of ^{14}C-UCB for delipidated HSA, derived from serial ultrafiltration of ^{14}C-UCB in solutions containing comparable HSA and chloride concentrations, after correction for the labeled degradation products of ^{14}C-UCB that passed the filter (9). This is valid, because the ultrafiltration studies had intentionally been performed at UCB/HSA ratios of 0.25 or below (9), at which binding of UCB to the second, lower-affinity site is insignificant (10). k_2 was calculated as $k_1/15 = k_2$ (10).

Most of the papers in the meta-analysis added the UCB ± HSA to cells incubated in protein-free DMEM, which has a total chloride concentration of 118 mM; two papers (20,21) used a chloride concentration of only 1 mM. Because the K_F values from the ultrafiltration studies had been obtained only in the presence of 50 mM chloride or no chloride, K_F values obtained at 50 mM or 0 mM chloride were applied, respectively. Based on published measurements of the affinity of chloride ions for delipidated HSA (18), these approximations may result in overestimation of K_F by, at most, 14 to 26% (see "Discussion").

All but three of the papers used unpurified UCB. Only the studies from the Lisbon group (19–21) used UCB that was purified by alkaline extraction of impurities and recrystallization from chloroform (22). All studies that included HSA, including the reference ultrafiltration studies of UCB-HSA binding, used delipidated HSA (Sigma Chemical Co, St. Louis, MO, U.S.A.). In all studies, the UCB had been dissolved in 0.1–1.0 N NaOH, then added to the buffered solution of HSA (if used), and then neutralized with HCl.

RESULTS

We report only comparisons of reported toxic effects of UCB with B_F levels calculated from the total UCB, HSA, and chloride concentrations provided in the published papers that were selected for the meta-analysis. We performed no direct measurements of the B_F levels in these media and no studies of the effects of UCB on cultured cells.

Studies with Unpurified UCB

Astroglial cells (Table 1). Cultured cerebral glial cells from rat embryos showed a significant decrease in mitochondrial function (MTT activity) when exposed for 2 h to B_F levels of 500 nM or higher (Fig. 1) (16). Trypan blue release by the same cells increased significantly at B_F levels of 1560 nM, but not at 119 nM; intermediate B_F levels were not tested. In contrast, cultured cerebral astrocytes from neonatal rats were damaged by $\geq$ 24-h exposure to B_F levels as low as 71 nM, exhibiting significant dose-related decreases in viability (increased LDH release) and mitochondrial function (MTT test) (Fig. 2) (23). A similar 24- to 48-h exposure of these cells to UCB likewise significantly increased LDH release, but the

Table 1. *Toxic effects of UCB on cultured rodent glial cells and astrocytes, with threshold B_F levels*

CNS region and cell type Animal species	UCB/HSA mol ratio	Cl^- (mM)*	Free UCB, B_F range (nM)	Exposure to UCB, (h)	Threshold B_F (nM)†	Function tested, change from control	Reference
Cerebral glial cells 20–21 day rat embryos	No HSA 0.5 or 2.0	NA 118	10–10,000 53–4800	2 1, 2	500 4800, 1560	MTT activity ↓ 41% Trypan blue release ↑ 8.7, 2.9×	16
Cerebral astrocytes 2-d-old neonatal rat	0.5–1.0 0.7–1.0	118	34–107 58–107	24–72 24–72	71 71	LDH release ↑ 1.8–3.3× MTT activity ↓ 48%	23
Cerebral astrocytes 2-d-old neonatal rat	1.7	118	194–26,818	24, 36, 48	721	LDH release ↑ 4.2, 11.1, 16.8×	24
Cerebral astrocytes 2-d-old neonatal rat	No HSA	NA	1,000–300,000	1 min	25,000	T_3 uptake ↓ 18%	15
Cortical astrocytes‡ 2-d-old neonatal rat	0.2–3.0 3.0	118	33–1050 6,060–29,807	0.25	309 6063§	[^{3}H]glutamate uptake ↓ 37% Apoptosis¶ ↑ 5×	19 20

* DMEM has total chloride concentration of 118 mM.
† Threshold B_F is the lowest unbound UCB concentration tested that produced a significant ($p < 0.05$) change from control.
‡ Only these studies used purified UCB.
§ Apoptosis was not studied at lower B_F.
¶ Hoechst stain for nuclear fragmentation was used.
Abbreviations used: NA, not available; ^{3}H-T, tritiated thymidine.

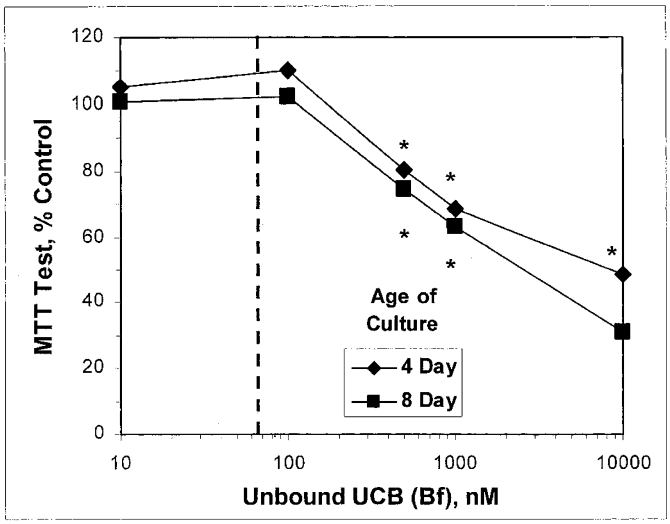

Figure 1. Effects on cultured embryonic rat cerebral glial cells of exposure for 2 h to 10 to 10,000 nM of unpurified UCB in the absence of albumin. Impairment of mitochondrial function (MTT test) was similar in cells cultured for 4 or 8 d. *$p < 0.05$ *vs* control. Derived from Figure 4 of Amit and Brenner (16).

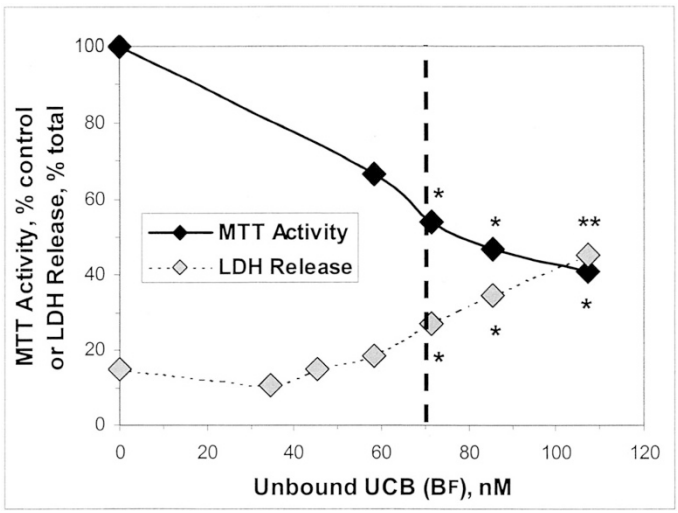

Figure 2. Effects on cultured neonatal rat astrocytes of exposure to varied concentrations of unpurified unbound UCB (B_F) for 24 h. A significant decrease in both mitochondrial function (MTT activity) and membrane integrity (increased LDH release) occurred at B_F levels at or above 71 nM, just above the aqueous solubility limit for UCB (70 nM, *vertical dashed line*). *$p < 0.05$, **$p < 0.01$. Derived from data of Chuniaud *et al.* (23).

threshold B_F level was 721 nM (Fig. 3), with no effect at 194 nM (24).

Another study with cultured brain astrocytes from newborn rats, performed without albumin, tested B_F values as low as 1 μM, but found no effect on the uptake of T_3 at B_F of 10 μM (15); at $B_F \geq 25$ μM, UCB caused dose-dependent inhibition of T_3 uptake, with a K_i of 31 μM. Biliverdin, bilirubin ditaurate and bilirubin glucuronides were progressively more effective inhibitors than UCB, supporting the concept that the inhibition was competitive and not a result of cytotoxicity.

Neuronal cells (Table 2). In the absence of HSA, MTT activity was impaired after 24 h of exposure of embryonic rat hippocampal neurons to 250 nM UCB ($p < 0.05$; figure not shown) (25) and of 14-d embryonic rat forebrain neurons to UCB concentrations as low as 400 nM (Fig. 4) (14) or 500 nM (data not shown) (13). The last two studies also demonstrated that exposure to 500 nM UCB for 24 to 96 h caused a large decrease in [^{3}H]thymidine incorporation, accompanied by increases in subsequent [^{3}H]thymidine release and in apoptosis. [^{3}H]leucine incorporation into cell protein was affected also after 6 h, with a triphasic response (13). A line of mouse neuroblastoma cells exposed to UCB/HSA systems for 22 h showed significant, dose-related decreases in MTT activity and [^{3}H]thymidine incorporation only at B_F levels of 775 nM and above (Fig. 5) (26). In a related paper from the same group (17), a line of rat neuroblastoma cells exhibited significant, multifunctional UCB toxicity after only 2–4 h of exposure to UCB/HSA systems, but only at B_F levels in excess of 1700

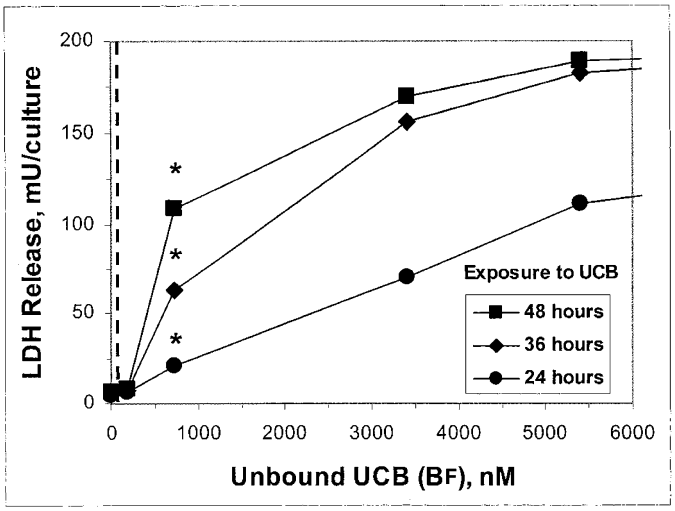

Figure 3. Effects on cultured neonatal rat astrocytes of exposure for 24, 36, or 48 h to concentrations of unpurified unbound UCB (B_F) ranging from 194 to 26,818 nM. *$p < 0.05$ at threshold B_F of 721 nM, as well as all higher concentrations. The effects above B_F of 6000 (not shown) are similar to those at 5400 nM. Derived from data of Rhine *et al.* (24).

nM; the true thresholds may have been much lower, because B_F levels between 400 and 1700 nM were not tested. The functions impaired included $^{42}K^+$ uptake, [^{3}H]thymidine incorporation into DNA, MTT activity, and incorporation of [^{35}S]methionine into cellular protein.

Studies with Purified UCB

Cortical astrocytes from neonatal rats showed a dose-related decrease in uptake of [^{3}H]glutamate after only 15 min of exposure to purified UCB at B_F levels above 300 nM (Table 1 and Fig. 6) (19). Apoptosis was observed also, but was studied only at B_F levels of 6063 nM or higher (20). Neonatal and embryonic cortical neurons from rats exhibited dose-related apoptosis when exposed for 4 h to B_F levels of 85 nM or higher (Table 2 and Fig. 7) (20, 21). At and above this threshold B_F level, the apoptosis was accompanied by release of cytochrome *c* from mitochondria, as well as activation of caspase-3, and cleavage of ADP-ribose polymerase (21). By contrast, other mitochondrial changes (translocation of Bax and collapse of membrane potential) were not observed until B_F levels reached 50 μM.

DISCUSSION

Applying the corrected affinity constants (K_F) (9), B_F exceeds maximum aqueous UCB solubility (70 nM) at B_T well below those at which the first binding site on HSA becomes saturated (Fig. 8*A*). At the normal adult HSA concentration of 600 μM, this occurs when B_T exceeds 80–85 μM (4.7–5.0 mg/dL; Fig. 8*B*). Except in Crigler-Najjar syndrome, B_T levels are rarely this high in adults with unconjugated hyperbilirubinemia. At the 25% lower mean albumin concentrations in newborn plasma (5), supersaturation would occur at B_T above 82 μM (>4.8 mg/dL; Fig. 8*B*), values commonly observed in uncomplicated neonatal hyperbilirubinemia. Thus, except possibly when HSA levels are low in jaundiced patients with cirrhosis, only neonates are exposed to plasma B_F levels above aqueous solubility.

At B_F levels above saturation, self-aggregation of UCB diacid must occur, progressing through three stages (7, 27–29). Oligomers of UCB diacid appear just above saturation; although they are too small to precipitate, they can dissociate reversibly and serve as a reservoir to replenish UCB monomers removed by cells. At higher UCB concentrations, larger colloidal aggregates form, stabilized by UCB mono- and dianions adsorbed on their surfaces (7). These microsuspensions may precipitate with prolonged standing (ripening) or neutralization of the charges by a decrease in pH (28). At yet higher UCB concentrations, coarser aggregates appear that precipitate spontaneously. Limited available data suggests that metastable aggregates are present at B_F as low as 1–2 μM at pH 7.0 to 7.4, but probably not at 500 nM (28, 29).

Our recalculated B_F levels for published *in vitro* studies (Tables 1 and 2) reveal that neurotoxic effects of even purified UCB can be observed at B_F levels ranging from slightly above to 11 times aqueous solubility (71–770 nM), although higher thresholds were obtained in some of the studies. Only a few of the original papers attempted to calculate or measure free bilirubin concentrations, and, when doing so, they used methods that have been shown to be flawed, as discussed elsewhere (8, 9). The variation of more than 10-fold among studies in our recalculated toxic thresholds for unbound bilirubin concentrations (Tables 1 and 2) is not unexpected, as the 12 papers used different cell systems from different species (26) of differing maturity, different cell functions, and different durations of culture (16) and exposure to UCB. Thus, the variable thresholds may simply reflect different susceptibilities of different cell systems to different types of injury.

Minor components in the DMEM, including those released by the cells themselves, might have influenced binding also. Although the different batches of delipidated HSA may have differed somewhat in their affinities for UCB, we have found that the binding affinity for UCB among four different batches of delipidated albumin from the same manufacturer (Sigma Chemical Co) varied by less than 4% (Ostrow JD, unpublished data). Thus, only a small error in B_F is introduced by our assumption that the binding affinity of the HSA used in our ultrafiltration study (9) is representative of the batches used in the studies in the meta-analysis.

Figures 2, 5, and 6 reveal a trend toward decreased viability or function at B_F levels below those at which a statistically significant impairment was attained. In all four cases, if those trends are assumed to be real effects, the resultant lower thresholds are still all slightly below (Fig. 2 and 6) to modestly above (Fig. 5) the solubility limit for unbound UCB. Some figures show gradual declines in function with increasing B_F levels, whereas in others, the threshold appears to be abrupt. Such differences, however, may be more apparent than real, depending on whether enough data points were obtained both above and below the true threshold.

Although, for reasons noted above, the thresholds varied with different studies and the responses were not uniform, our findings clearly establish that that marked supersaturation and precipitation of UCB are not necessary to produce toxicity to

Table 2. *Toxic effects of UCB on cultured rodent neuronal cells, with threshold B_F levels*

CNS region and cell type Animal species	UCB/HSA mol ratio	Cl^- (mM)*	Free UCB, B_F range (nM)	Exposure to UCB (h)	Threshold B_F (nM)†	Function tested, change from control	Reference
Hippocampal neurons 17-d embryonic rat	No HSA	NA	25–250	24	250	MTT activity ↓ 26%	25
Forebrain neurons 14-d embryonic rat	No HSA	NA	250–5000	72 96	400 500	MTT activity ↓ 13% Apoptosis (DAPI) ↑ 5×	14
Forebrain neurons 14-d embryonic rat	No HSA	NA	500 only	48, 72, 96 96 24, 96 24, 96	Only 500 tested	MTT activity ↓ 18, 21, 26% Apoptosis (DAPI) ↑ 6.8× [^{3}H]leucine incorp. ↓ 39, 41% 2-DG uptake ↓ 66, 35%	13
Neuroblastoma cells mouse (NBR10A line)	0.8–1.5	118	100–3900	22	775‡ 786‡	MTT activity ↓ 41% ^{3}H-T incorporation ↓ 58%	26
Neuroblastoma cells rat (N115 line)	0.8, 1.5 1.5 only	118 118	301–383 and 1766–2378 2113 only 2113 only	2, 4 2, 4 1–4 1–4	1766 2378 Only 2113 tested	$^{42}K^+$ uptake ↓ 26% (4 h only) ^{3}H-T incorporation ↓ 23, 50% MTT activity ↓ 13–63% [^{35}S]methionine incorp. ↓ 23–57%	17
Cortical neurons§ neonatal and embryonic rat	0.5–3.0	1	85–2900	4	85	Apoptosis‖ ↑ 2×¶	20 21

* DMEM has total chloride concentration of 118 mM.

† Threshold B_F is the lowest unbound UCB concentration tested that produced a significant ($p < 0.05$) change from control.

‡ Rat neuroblastoma cell line N115 was tested also and showed no effect on MTT activity until B_F was 2600, but virtually identical sensitivity to the mouse line when [^{3}H]thymidine incorporation was tested.

§ Only these studies used purified UCB.

¶ Release of cytochrome *c* from mitochondria, as well as activation of caspase-3, and cleavage of ADP-ribose polymerase were demonstrated also at the same threshold B_F level.

‖ Hoechst stain for nuclear fragmentation was used.

Abbreviations used: NA, not available; DAPI, 4′, 6-diamidino-2-phenylindole; 2-DG, 2-deoxyglucose; ^{3}H-T, tritiated thymidine.

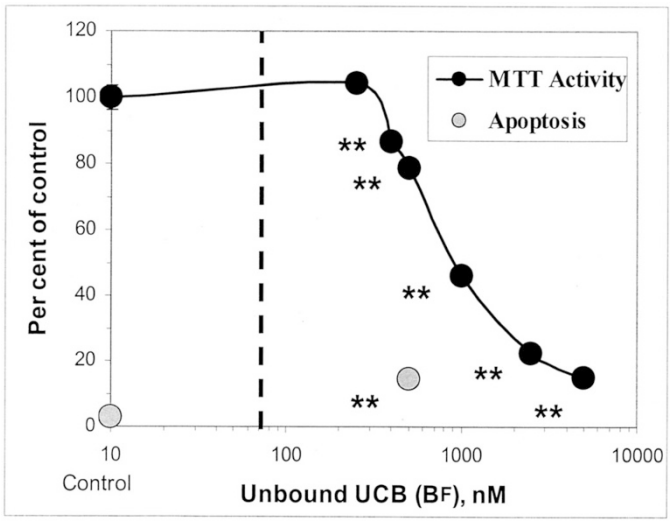

Figure 4. Effects on cultured forebrain neurons from 14-d-old rat embryos of exposure for 72–96 h to varied concentrations of unpurified unbound UCB (B_F). A significant decrease in mitochondrial function (MTT activity) was observed at B_F levels at or above 400 nM, and an increase in apoptosis at a B_F level of 500 nM, 5.7 and 7.1 times, respectively, the aqueous solubility limit for UCB (70 nM, *vertical dashed line*). $^{**}p < 0.01$. Derived from data of Grojean *et al.* (14).

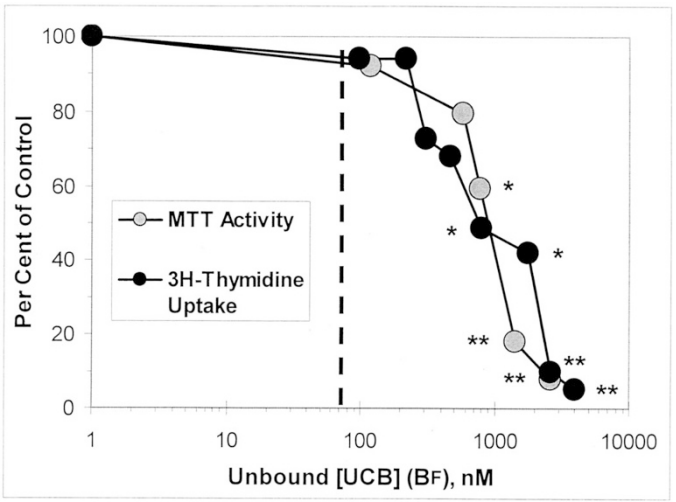

Figure 5. Effects on cultured mouse neuroblastoma cells (NBR10A line) of exposure for 22 h to varied concentrations of unpurified unbound UCB (B_F). A significant decrease in both mitochondrial function (MTT activity) and [^{3}H]thymidine uptake occurred at B_F levels at or above 780 nM, 11 times the aqueous solubility limit for UCB (70 nM, *vertical dashed line*). $^*p < 0.05$, $^{**}p < 0.01$. Derived from data of Schiff *et al.* (26).

CNS cells. This renders untenable the long-accepted concept that only coarse UCB aggregates, which may include coprecipitated albumin, are involved in early UCB toxicity (7). Even allowing for potential moderate inaccuracies in our calculated values of unbound bilirubin (9), our findings strongly suggest that toxicity develops only near or above the aqueous saturation limit of 70 nM, a range in which only UCB monomers, soluble oligomers, and metastable small colloids are likely to be present. By contrast, B_F levels well below 70 nM [aqueous

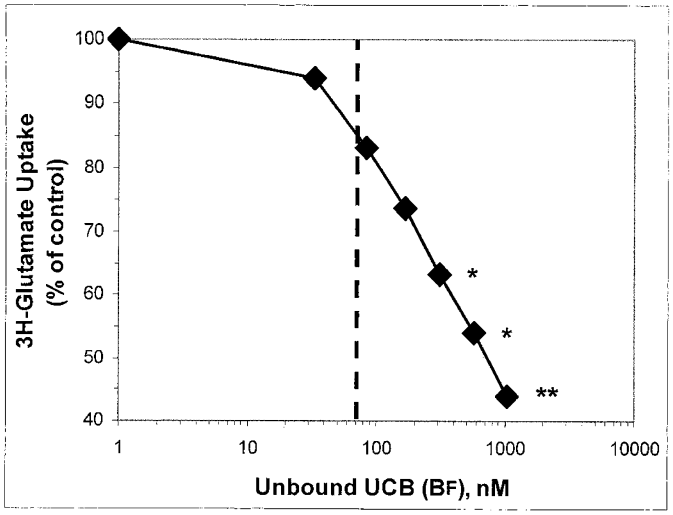

Figure 6. Effects on cultured cortical astrocytes from 2-d-old neonatal rats of exposure for 15 min to varied concentrations of purified unbound UCB (B_F). A significant decrease in [^{3}H]glutamate uptake was observed at B_F levels at or above 309 nM, 4.4 times the aqueous solubility limit for UCB (70 nM, *vertical dashed line*). *$p < 0.05$, **$p < 0.01$. Derived from data of Silva *et al.* (19,20).

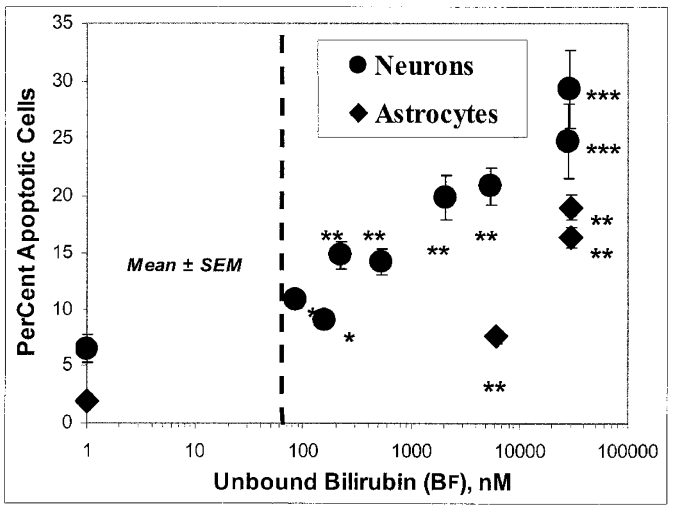

Figure 7. Effects on cultured cortical neurons and astrocytes from neonatal and embryonic rats of exposure to varied concentrations of purified unbound UCB (B_F) for 4 h. A significant increase in apoptosis of neurons occurred at B_F levels at or above 85 nM, slightly above the aqueous solubility limit for UCB (70 nM, *vertical dashed line*). Astrocytes were affected at 6063 nM, but lower B_F levels were not tested. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Derived from data of Silva *et al.* (20) and Rodrigues *et al.* (21).

saturation (30)] appear to protect CNS cells against oxidative damage (25, 31), and this protection is lost because of the countervailing toxic effects of UCB at higher B_F levels (25).

At these relatively low B_F levels, both astrocytes and neurons were susceptible to impairment of mitochondrial functions (MTT activity and apoptosis), whereas diminished incorporation of [^{3}H]thymidine was reported only for neurons. These toxic effects can account for the structural features of apoptosis that appear in the cerebellum and cochlear nucleus of jaundiced Gunn rat pups (32–34) and in the basal ganglia of kernicteric human infants (5). These early changes appear well before

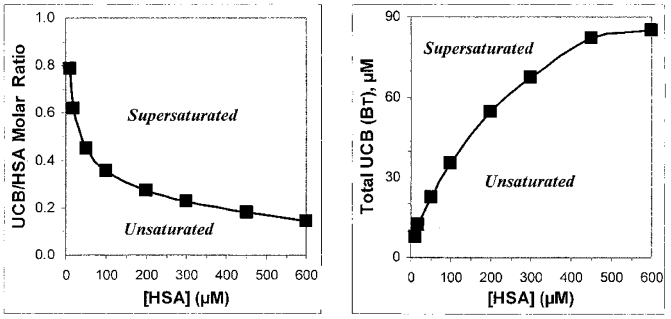

Figure 8. UCB/HSA ratios (*left*) and total UCB concentrations (*right*) at which calculated B_F values equal the aqueous solubility of unbound UCB at pH 7.4 over a range of albumin (HSA) concentrations. Areas above each curve are supersaturated with unbound UCB, whereas areas below the line are unsaturated. At mean normal plasma albumin concentrations of 450 μM for neonates and 600 μM for adults, supersaturation occurs at UCB/HSA ratios as low as 0.2 and at total UCB concentrations above 80 μM. B_F values were calculated by applying corrected affinity constants of UCB for HSA in the presence of chloride (9) at the albumin concentrations indicated. The calculations (equation 1) assumed independent binding of UCB to two sites on albumin (10).

peak UCB levels are attained, but ultimately progress to atrophy of these CNS regions.

The modestly supersaturated B_F levels also affected astrocyte membranes, as shown by increased LDH release (23) (Fig. 2) and impaired [^{3}H]glutamate uptake (Fig. 6) (19). The extremely brief period of exposure to UCB probably explains why Warr *et al.* (35) did not detect changes in glutamate transporters, *N*-methyl-D-aspartate receptors, or electrical currents in retinal glial cells from salamanders after treatment with 10 μM UCB for only 10–50 s.

Effects of purified UCB on the membrane structure of neurons (21) and mitochondria (20) have been observed, however, only at highly supersaturated B_F levels in the micromolar range, at which UCB precipitation is expected. This fits with historic concepts that precipitation of UCB in cell membranes alters membrane fluidity and the activity of integral membrane proteins (7), but it is problematic as to whether this is relevant to the modestly elevated B_F levels associated with the reversible stages of bilirubin encephalopathy. Monomers of UCB diacid cannot penetrate deeply into membranes (36), but bind near the surface of the outer leaflet of the membrane (37–39). The resultant modest perturbation of membrane structure might be a factor in the early cellular toxicity of clinically relevant concentrations of UCB (39).

Ahlfors (8) applied a peroxidase-diazo method to reassess B_F for historic data on plasma B_T and HSA concentrations in jaundiced neonates and concluded that kernicterus was likely only when B_F levels exceeded 60 nM (40), in apparent agreement with our results for *in vitro* systems. There are, however, important differences between plasma or serum and *in vitro* systems that limit comparisons between his study and ours. Plasma contains additional proteins that bind UCB, such as apo D (41), so that B_F levels in plasma are lower than those in solutions containing the same concentration of purified albumin (42). On the other hand, FFA and other substances not present in defined solutions containing delipidated albumin may inhibit the binding of UCB to albumin (7). Finally, *in vivo*,

neurons and astrocytes are not exposed directly to plasma, but are separated by the blood–brain and blood–cerebrospinal fluid barriers that may limit penetration of unbound UCB into the CNS (6). Thus, the media to which the CNS cells are exposed *in vitro* are the equivalent of the cerebrospinal fluid and extracellular fluid in the brain, where, in jaundiced Gunn rats, total UCB concentrations may be only one fifth those in plasma (43) and albumin concentrations are much lower than plasma. Overall, therefore, the threshold B_F levels for UCB neurotoxicity are likely to be higher in plasma *in vivo* than in defined albumin solutions *in vitro*.

CONCLUSIONS

Because of the above-noted differences between *in vitro* and *in vivo* systems, as well as interspecies differences, it remains to be determined whether, to fully prevent bilirubin encephalopathy, treatment of neonatal hyperbilirubinemia should be instituted at plasma UCB levels lower than those that are currently recommended (5, 44). Nonetheless, our findings favor a role for small, soluble UCB aggregates, present at moderately supersaturated B_F levels, in the often-reversible damage to mitochondria, and possibly plasma membranes of CNS cells that characterize the early stages of bilirubin encephalopathy.

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