Clearance of Bilirubin from Rat Brain after Reversible Osmotic Opening of the Blood-Brain Barrier

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ABSTRACT. Utilizing multicomponent spectrophotometry, we assayed the bilirubin content of rat cerebral hemispheres. With this assay, we determined the clearance of bilirubin from the rat brain following reversible, osmotic opening of the blood-brain barrier. Clearance was rapid, with a half-time of 1.7 h. This half-time was the same as that for clearance of bilirubin from the serum, suggesting that brain bilirubin was removed by transport or diffusion back into the general circulation. Osmotic opening does not damage brain tissue. Thus, in the undamaged rat brain, bilirubin is rapidly cleared, in contrast to its persistence in autopsy-proven human kernicterus. The potential for clearance of bilirubin from human neonatal brain should be considered, especially in the absence of underlying tissue damage. (*Pediatr Res* 19: 1040-1043, 1985)

Hyperbilirubinemia is likely the most common medical diagnosis in the newborn. Physicians treat neonatal jaundice in order to prevent the neurotoxicity associated with brain uptake of bilirubin (kernicterus). Research on the cerebral transport and toxicity of bilirubin continues, but the results remain unclear (1). Uncertainty extends to clinical practice as well. For example, the preceding edition of *Hospital Care of Newborn Infants*, published by the American Academy of Pediatrics, included guidelines for exchange transfusion (2). The current edition omits guidelines, noting a diversity of approaches among physicians (3). In part, this diversity results from uncertainty about how bilirubin enters the brain and the anatomic and biochemical loci of bilirubin toxicity.

The blood-brain barrier normally restricts exchange of water-soluble substances and proteins between blood and brain, but is damaged in diseases such as hypertension, by ischemia or trauma. Experimentally, the blood-brain barrier can be unilaterally and reversibly opened without causing brain damage by infusing a hypertonic solution of arabinose into one of the carotid arteries (4). Permeability returns to normal within about 1 h after arabinose infusion (5). We have previously shown that circulating albumin-bound bilirubin enters the side of the rat brain receiving the arabinose (4, 6). Furthermore, the regional cerebral distribution of albumin mimicks that seen in human kernicterus, providing a potential model for the human disease.

We designed this study to determine the time-course of bilirubin entry into and removal from the brain after osmotic opening of the blood-brain barrier. For the study, we also developed rapid, simple techniques for the extraction and quantitation of bilirubin in brain tissue.

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MATERIALS AND METHODS

The blood-brain barrier was opened reversibly as previously described (4, 6), using 3-month-old, male Osborne-Mendel rats weighing 250 to 350 g. In the current study, we wished to achieve higher brain bilirubin concentrations, and so the albumin-bilirubin solution was infused directly into the carotid catheter, after hypertonic arabinose treatment. The carotid route gives about a 10-fold increase in brain content of the infused substance, compared to peripheral, intravenous delivery (7).

The blood-brain barrier was opened in one cerebral hemisphere by infusing a 1.8 molal arabinose solution (warmed to 37° C) for 30 s, at a flow rate of 7.2 ml/min, into one carotid artery. This procedure has been described in detail (5). One min after termination of the arabinose infusion, a 15-min, constant-rate infusion of a bilirubin-albumin solution was started through the same carotid catheter, for a total volume of 10 ml/kg (6). Arterial blood was sampled at various times for the assay of serum bilirubin. Animals were killed at 0.27, 0.50, 1, 3, 5, 9, or 18 h after initiation of arabinose infusion, by intravenous injection of sodium pentobarbital. The cerebral circulation was then cleared of blood by aortic perfusion with isotonic saline (6). The brain was removed, and the cerebral hemispheres were dissected separately and homogenized.

Two sets of control animals were also studied. All received the arabinose infusion through a femoral catheter, which has no effect on the blood-brain barrier (3, 6). Half of the animals received the bilirubin infusion via a femoral catheter (control F) and half through the carotid artery (control C). Controls were killed at 0.50 h, at which time brain bilirubin was maximal in the experimental animals.

Bilirubin-albumin solution for infusion. The isotonic, neutral solution contained 5 g/l bilirubin (8.55 mM) and 280 g/l bovine serum albumin (4.21 mM). It was stable for at least 2 wk, after which a fresh solution was made. Bilirubin and sterile 0.85% sodium chloride and 350 g/l serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, product A-5128). We confirmed the stated concentration of the albumin (8). The solution was made as follows. A solution of 25 mg/ml bilirubin was prepared in 125 mM NaOH. Using a Millex 0.22 µ GV filter (Millipore Corp., Bedford, MA), 2.5 ml bilirubin solution was then added to each 10 ml of albumin solution in the sterile vial containing the albumin. The solution was stored at 4° C, covered with foil to protect from ambient light. When withdrawing an aliquot for carotid infusion, a Millex GV filter was again interposed. A final Millex filter was also placed in-line on the carotid catheter to assure removal of any precipitated bilirubin/albumin which might result in embolic infarction (9).

Homogenization and extraction. After the brain was removed, the individual hemispheres were weighed in tared 12 × 75 mm polypropylene tubes (Falcon no. 2063, Oxnard, CA). The water content was held constant at 1.25 ml, to assure quantitative

recovery of bilirubin (see "Results"). Taking the brain water content to be 79% (5),

$$(\text{volume to add, ml}) = 1.25 - 0.79 \times (\text{weight, g})$$

The contents were homogenized by sonication with a Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Plainview, NY) equipped with a microtip, at the no. 7 setting, for two 15-s periods, separated by a 15-s interval. The extraction mixture was that suggested by Berry (10), but modified to approach the azeotropic composition (11): 2 vol chloroform/1 vol methanol/5 mg/ml cetyltrimethylammonium bromide (Sigma). After adding 3 ml of the solution, the tube was capped, shaken well, vortexed, and then centrifuged for 10 min in a table-top centrifuge. The bilirubin was in the lower, organic phase in a volume of 2.14 ml. To recover this solution, the cap was removed, and the bottom of the tube was punctured with a 21-g needle. The bilirubin solution was collected in a 1.5-ml polypropylene centrifuge tube. Complete recovery was not essential because the concentration of bilirubin was determined spectrophotometrically. Extracts not assayed immediately may be stored at -20°C in the dark for a minimum of 2 wk without affecting the assay. Occasionally, particulate matter was carried over with the organic solvent. This could be removed by 3-min centrifugation at $12,000 \times g$ (Microfuge, Beckman Instruments, Fullerton CA), but removal was not necessary because the bilirubin concentration and the recovery were unaffected.

Spectrophotometric determination of bilirubin and Hb. Bilirubin frequently is assayed spectrophotometrically, but Hb interferes with this assay. The absorbance due to Hb therefore must be subtracted (12, 13) or the Hb must be removed. Therefore, we utilized a spectrophotometric method which simultaneously quantitates bilirubin and Hb in the solution.

Multicomponent analysis (8, 14–16) was applied to data obtained with a computerized spectrophotometer (Hewlett-Packard model 8450A, Palo Alto, CA). The spectrum of the brain extract was first measured against a solvent blank. Then the built-in multicomponent analysis routine was utilized, using the first derivative, from 404 to 550 nm. Spectral standards were bilirubin and human Hb, dissolved in the extraction solvent. The molar absorptivity of bilirubin in the solvent was 57,400, compared to 61,100 in chloroform (17). The spectrophotometer determined the concentration of bilirubin and of Hb in the extract in a few seconds. The concentration of bilirubin in the original brain was then calculated, assuming a density of 1.0 for the brain tissue (18):

$$[\text{brain bilirubin, } \mu\text{M}] = \frac{[\text{extracted bilirubin, } \mu\text{M}] \times 2.14}{(\text{brain weight, g})}$$

Recovery, assessed by adding known amounts of bilirubin to cerebral hemispheres from rats not exposed to bilirubin, was 99.0%.

RESULTS

Extraction of bilirubin. Extraction of bilirubin from tissues or fluids can permit its determination by simple spectrophotometric assay. Organic solvents often are used for extraction, with the assumption that interfering compounds such as heme are not extracted (10, 19). This assumption is not strictly correct. As illustrated in Figure 1, 19% of a 6 μM solution of Hb was extracted by the methanol/chloroform extraction solvent. Accurate assessment of bilirubin in brain, corrected for Hb content, can be obtained as described in "Materials and methods", by fitting over a wide portion of the absorption spectrum, using multicomponent analysis. Even massive contamination with heme does not interfere with accurate determination of bilirubin by this method (Table 1).

Chloroform, methanol, and water form a constant boiling azeotrope. The lower layer, which contains the bilirubin, will be 83% chloroform, 14% methanol, and 3% water (11). Thus, the amount of water in the extraction system could affect the efficiency of extraction. As shown in Figure 2, the recovery of

Table 1. Effect of added Hb on the quantitation of bilirubin*

Bilirubin (μM)	Hb (μM)	Measured absorbance		Measured bilirubin (μM)
		450 nm	402 nm	
1.4	0.00	0.088	0.057	1.39
1.4	0.16	0.093	0.078	1.39
1.4	0.31	0.091	0.092	1.36
1.4	0.47	0.096	0.110	1.38
1.4	0.62	0.097	0.127	1.35
1.4	0.78	0.094	0.129	1.33
2.9	0.00	0.187	0.119	2.93
2.9	0.16	0.191	0.139	2.93
2.9	0.31	0.197	0.162	2.94
2.9	0.47	0.189	0.167	2.87
2.9	0.62	0.195	0.188	2.87
2.9	0.78	0.191	0.193	2.84

* Bilirubin solutions were prepared with increasing concentrations of Hb. These solutions were extracted and the bilirubin concentrations were determined as described in "Materials and Methods."

Fig. 1. Spectrum of 6 μM solution of human Hb, extracted as described in "Materials and Methods." Nineteen percent of the Hb was extracted.

Fig. 2. Effect of added water on the recovery of bilirubin in the extraction solvent. With no added water, the volume of the bilirubin-albumin solution was 1.0 ml. The solution was extracted with 3.0 ml solvent as described in "Materials and Methods."

Table 2. Bilirubin concentrations in brain and serum*

Time after arabinose (h)	n	Serum (μ M)	SEM (μ M)	Right hemisphere (μ M)	SEM (μ M)	Left hemisphere (μ M)	SEM (μ M)
0.27	10	883	16	38.07	9.61	10.74	2.52
0.50	10	636	19	43.15	11.23	6.47	0.97
1.00	10	517	44	50.69	12.86	10.97	2.58
3.00	9	199	25	12.81	2.82	6.18	1.86
5.00	10	87	6	10.33	1.97	2.80	0.53
9.00	8	70	8	3.00	0.33	1.99	0.34
18.00	4	31	8	1.28	0.18	0.99	0.20
0.50 (control F)	5	578	64	1.56	0.17	2.15	0.37
0.50 (control C)	5	681	29	9.40	2.49	6.19	1.37

* As described in "Materials and Methods," the blood-brain barrier was opened osmotically by infusion of arabinose into the right carotid artery of the experimental animals. This was followed 30 s later by infusion of the bilirubin-albumin solution. Both sets of control animals received the arabinose infusion through a femoral venous catheter, so that their blood-brain barriers remained intact. Control F animals received the bilirubin through the femoral venous catheter while control C animals received the bilirubin through the carotid arterial catheter. Within each column, concentrations did not differ significantly at 0.27, 0.50, and 1.00 h ($p > 0.05$ by Student's t test).

bilirubin initially remained constant when additional water was added and then fell when added water exceeded 40% of the initial volume. For this reason, the water content of the brain was taken into account when extracting bilirubin (see "Materials and methods").

Clearance of bilirubin by the rat brain. Circulating bilirubin entered the brain, presumably as the bilirubin-albumin complex, when the blood-brain barrier was opened unilaterally by carotid infusion of hypertonic arabinose (Table 2). The initial serum concentration and clearance of bilirubin matched values reported earlier (6) following femoral venous infusion of the bilirubin-albumin solution. Thus, carotid infusion of the bilirubin-albumin solution did not affect clearance of bilirubin from the blood.

Ipsilateral brain bilirubin content increased dramatically following carotid infusion of hypertonic arabinose, but not after carotid infusion of isotonic saline or after femoral venous infu-

sion of hypertonic arabinose (Table 2). Brain concentrations reached their maximum within 1 h and then fell rapidly. Figure 3 is a semilogarithmic plot of brain and serum concentration versus time, permitting comparison of their clearances. Following the initial distribution phase, the lines were parallel. The half-times for clearance were the same: the half-time for serum was 1.6 h and that for brain was 1.7 h. Thus, bilirubin that entered the rat brain following opening of the blood-brain barrier was rapidly cleared.

DISCUSSION

This study reports a simple and rapid technique for the extraction and quantitation of bilirubin within brain. Sonication provides a particularly fast method to homogenize brain tissue. We were not able to develop solvents which could extract bilirubin alone. This was true of the organic solvent systems as well as denaturing salts such as guanidine hydrochloride. Following extraction, bilirubin should be quantified with a sensitive assay unaffected by contaminating compounds such as heme. Alternatively, bilirubin should be separated from the contaminants by a technique such as high-pressure liquid chromatography. Multicomponent analysis of the visible region spectrum proved sensitive and rapid. This technique appears capable of quantitating several components in a solution, even with extreme spectral similarity (8, 14). Our preliminary studies suggest that the multicomponent analysis is also useful for determination of bilirubin in serum and amniotic fluid without prior extraction.

Several routes of clearance from brain are possible. First, the bilirubin could be metabolized *in situ*. Brodersen and Bartels (20) demonstrated that rat brain is rich in a bilirubin oxidase capable of converting bilirubin to colorless products (20). Second, brain bilirubin (presumably bound to albumin) could be cleared by movement into the cerebrospinal fluid ("sink effect"). Bilirubin and albumin do appear in the cerebrospinal fluid of animals; the concentrations are elevated in neonatal jaundice (21, 22). Third, bilirubin could be cleared by transport or diffusion back into the general circulation via the cerebral capillaries. The parallel clearance of brain and serum bilirubin makes this the likely major mechanism of clearance (Fig. 3). The mechanism remains to be studied, but transport of organic anions is well established.

From experience with neonatal kernicterus, many clinicians have concluded that bilirubin is retained by the brain and that staining is not readily reversed. This may be true if brain tissue

Fig. 3. Clearance of bilirubin from serum (+) and right-left cerebral hemisphere (□). To permit comparison on the same scale, the concentrations were converted to a percentage of the maximal concentration. Note the semilogarithmic plot. Within each line, the concentrations at 0.27, 0.50, and 1.0 h do not differ significantly ($p > 0.05$).

is dead or damaged, because bilirubin, like many organic dyes, should preferentially bind to damaged tissue (23). However, osmotic opening of the blood-brain barrier does not damage tissue. With this technique, we demonstrated a rapid clearance of bilirubin from the undamaged adult rat brain. The potential for rapid clearance of bilirubin from neonatal human brain exists, provided the brain is otherwise undamaged. Damaged tissue, which also might be edematous, may be more likely to bind bilirubin and is therefore vulnerable to the toxicity of bilirubin (21).

The possible implication of the blood-brain barrier in kernicterus was noted decades ago (24). In this study, we utilized osmotic opening of the blood-brain barrier to augment entry of blood-borne bilirubin into the adult rat brain. Osmotic opening is reversible, does not cause brain damage, and has been studied in detail (4, 5). Blood-brain barrier permeability is essentially normal within 1 h after arabinose infusion. Osmotic treatment also transiently decreases cerebral blood flow, but increases cerebral glucose utilization and brain water content (25). Thus, metabolic activity is increased while blood flow is reduced. We speculate that these effects might render the brain more susceptible to toxic effects of bilirubin.

Osmotic treatment does cause the transient metabolic effects mentioned, and it would be difficult to distinguish these effects from those of bilirubin. Thus, one should wait 2 h after arabinose treatment to assure full reversal of any effects of the osmotic treatment (5, 25). Infusion of bilirubin through the carotid catheter loads the brain much more effectively than does peripheral venous infusion. As a result, sufficient bilirubin remains after 2 h to permit the study of its effects.

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