Blood-Brain Barrier Permeability to Bilirubin in the Rat Studied Using Intracarotid Bolus Injection and *in Situ* Brain Perfusion Techniques

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ABSTRACT. It has generally been assumed that free unconjugated bilirubin gains access to the brain because of its lipid solubility. However, no measurements of the blood-brain barrier permeability to free bilirubin exist. The aim of these experiments was to determine the blood-brain barrier permeability in the rat to free bilirubin using single-pass (Oldendorf) and *in situ* perfusion (Takasato) techniques. Studies were performed on adult rats under sodium pentobarbitone anesthesia. [^3H]bilirubin IX- α -ZZ (sp act 6.2 Ci/mmol) was synthesized by reduction of biliverdin with sodium boro- ^3H hydride. Blood-brain barrier permeability to albumin-bound and free bilirubin was determined using injectates/perfusates containing a molar excess of human serum albumin with or without the addition of the displacing agent sulphadimethoxine. For free bilirubin, the brain uptake index was 28.5 ± 9.3 (mean $\pm$ SD, $n = 18$), and the permeability surface area product was $54.84 \pm 36.38 \times 10^{-4}$ mL/s/g (mean $\pm$ SD, $n = 13$). These results demonstrate that the behavior of free bilirubin *in vivo* in relation to the cerebral microvasculature corresponds to that of a "lipid soluble" molecule. (*Pediatr Res* 27: 436-441, 1990)

Abbreviations

BUI, brain uptake index

PA, permeability surface area product

In humans and other mammals, metabolism of the heme component of Hb generates the linear tetrapyrrole bilirubin. In man, the naturally occurring form of this compound is bilirubin IX- α -ZZ. It circulates bound to albumin and, under physiologic conditions, the concentration of unbound bilirubin is very low. Conjugation by esterification with glucuronyl groups in the liver allows its excretion in bile.

Serum levels of unconjugated bilirubin frequently rise above normal in human newborns. This neonatal jaundice is a concern in the medical care of newborn infants because of its association with neurotoxicity. The bilirubin encephalopathy associated with high serum levels of bilirubin implies that this molecule gains access to the CNS.

The mode of entry of bilirubin into the brain remains uncertain. Initially, it was suggested that the susceptibility of the newborn to bilirubin neurotoxicity reflects the increased permeability of an "immature" blood-brain barrier. However, available

evidence indicates that the blood-brain barrier in humans is "tight" to macromolecules from an early stage in gestation (1).

It is now generally assumed that bilirubin crosses the blood-brain barrier because of its lipid solubility, but there is no direct evidence for this contention. Although intramolecular hydrogen-bonding masks polar groups and drastically reduces the aqueous solubility of bilirubin, especially when protonated as the acid, the molecule has very low solubility in nonpolar solvents such as ether (2). It is therefore not lipid soluble in the generally accepted sense. However, there is no doubt that it does have a high affinity for membrane lipids (2).

Diamond and Schmid (3) attempted to measure permeability of the blood-brain barrier to free bilirubin, and their study is generally cited as evidence for the "free bilirubin" hypothesis. However, these experiments did not include measurement of free bilirubin levels, and no attempt at quantitation was made.

The aim of our experiments was to quantify the transfer of bilirubin from blood to brain in the adult rat under conditions in which the relative proportions of free and albumin-bound bilirubin could be controlled and defined. The intracarotid bolus injection and *in situ* brain perfusion techniques were used. Evidence was obtained that the blood-brain barrier in the cerebral hemisphere of the adult rat is permeable to free bilirubin.

MATERIALS AND METHODS

Surgical Procedure. Studies were performed on adult male rats (Wistar strain), body wt 300–500 g. The rats had free access to a standard diet and water before surgery. Anesthesia was induced with halothane (Fluothane, ICI, Macclesfield, England), and maintained with sodium pentobarbitone (Sagatal, May & Baker Ltd, Dagenham, England) given initially as an intraperitoneal dose of 50 mg/kg body wt and thereafter intravenously at intervals. The left femoral artery and vein were cannulated, and arterial blood pressure was monitored continuously. Tracheostomy was performed and intermittent positive pressure ventilation instituted to maintain normal respiratory blood gas tensions. The right external carotid artery was exposed and clamped proximally during surgery. After ligation of the superior thyroid branch, it was retrogradely cannulated with polythene tubing (Portex, Hythe, England; outer diameter 0.8 mm, inner diameter 0.4 mm, length 15 cm) such that the tip was sited 2–4 mm distal to the bifurcation of the common carotid artery (Fig. 1a). Care was taken not to interrupt flow to the right internal carotid at any time during surgery. Cannulae were kept patent with heparinized normal saline (2 IU sodium heparin/mL saline). In the case of *in situ* brain perfusion studies, the right common carotid artery was prepared for ligation by loosely encircling the artery with surgical thread (Fig. 1b). Surgery was performed on a heated operating table adjusted to maintain the animal's rectal temperature at $37.0 \pm 0.5^\circ\text{C}$. Uptake studies were terminated by decapitation at 5 s in single-pass studies and at 60 s in brain perfusion

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Fig. 1. External carotid artery cannulation for (a) single-pass studies in which the injectate bolus is carried through the cerebral circulation by uninterrupted common carotid artery flow and (b) *in situ* perfusion where the common carotid artery is ligated immediately before infusion.

experiments using a guillotine (Harvard Instruments, Boston, MA).

Radiochemicals. Butanol, N-[1- 14 C]-(sp act 1.0 mCi/mmol, New England Nuclear, Boston, MA). 99m Tc as sodium pertechnetate (Elumatic III 99m Tc generator, International-CIS, St. Quentin-Yvelines Cedex, France). D-[U- 14 C]-alanine (sp act 20 mCi/mmol, Amersham International Corp., Arlington Heights, IL). Bilirubin of sufficiently high sp act was not available commercially. [3 H]Bilirubin IX- α (sp act 6.2 Ci/mmol) was therefore specially prepared in collaboration with Amersham International's tritium-labeling service (TR5), as follows: crystalline bilirubin (Sigma Chemical Co., St. Louis, MO, cat. no. B4126) was purified by the method of McDonagh and Assisi (4), and used in the preparation of purified biliverdin as described by McDonagh and Palma (5). Purified biliverdin (4 mg dissolved in 1 mL methanol) was reduced with sodium borohydride (0.6 mg solid, sp act 50 Ci/mmol), using an adaptation of the method described by Hutchinson *et al.* (6). The reagents were stirred in darkness under nitrogen at room temperature for 15 min. The reaction was halted with 10 mL glycine hydrochloride buffer, pH 2.8, and the product extracted with chloroform (10 mL $\times$ 3). Unincorporated tritium was removed by rotary evaporation after the addition of methanol. The product was pumped to dryness, sealed under argon in a glass ampoule, and transported to University College, London at -20°C . The [3 H]bilirubin was retrieved in chloroform (15 mL) and reduced in volume to 0.8 mL before purification by thin-layer chromatography to remove the isomers III- α and XIII- α (Merck TLC silica gel (H) plates activated at 120°C for 60 min, and developed under argon with 1% glacial acetic in analytical reagent chloroform). The bilirubin IX- α band was eluted from the plate and extracted from the silica gel by washing with chloroform and centrifugation ($\times$ 3). [3 H]Bilirubin IX- α (sp act 6.2 Ci/mmol) was stored dry in aliquots of 0.25–2.0 nmol, under argon in sealed ampoules at -20°C in the dark.

Preparation of Injectates/Perfusates. Despite the optimal storage conditions used, the radiochemical purity of stock [3 H]bilirubin had declined to 90% at the time of these experiments. After the addition of a 20-fold M excess of albumin to effectively bind all bilirubin, approximately 10% of the tritium activity remained dialyzable, indicating that it was not associated with bilirubin. A further purification process was therefore adopted immediately before its use. [3 H]Bilirubin was retrieved from storage ampoules in a solution of 0.1 M sodium hydroxide and 5 mM EDTA, 0.4–0.8 mL. Human serum albumin (Kabi, Uxbridge, England), 0.8–1.6 mL, at pH 9 to prevent aggregation of bilirubin, was added in 20-fold M excess. The pH was adjusted

to between 7.5 and 8.0 by addition of 0.5 M HCl. Unincorporated tritium was then removed by membrane dialysis against Ringer's saline, pH 7.5, gently agitated for 18 h at room temperature, in the dark. Visking tubing with a mol wt cutoff of 12 000–14 000 was used to dialyze 1 vol of albumin-bound bilirubin against 1 000 vol Ringer's saline. The use of albumin-binding to facilitate purification made it necessary to add a displacing agent to study free bilirubin uptake. The sulfonamide sulfadimethoxine (Madrison, Roche, Welwyn Garden City, England), which displaces bilirubin from its primary binding site on albumin, was used.

Sufficient dialyzed [3 H]bilirubin was prepared to perform up to six experiments. Half was used to study the uptake of albumin-bound bilirubin, and sulfadimethoxine was added to the remainder for free bilirubin studies. Sulfadimethoxine was added at a concentration of 10 mmol/L to injectates and 5 mmol/L to perfusates. The efficiency of the purification process and the amount of [3 H]bilirubin displaced from albumin by the sulfonamide were determined by centrifugation of samples of the injectate/perfusate, at the time of individual uptake studies, using Centriflo membrane cones (type CF25, Amicon Corp., Danvers, MA) operating below the maximum recommended relative centrifugal force of $1000 \times g$, at room temperature.

The pH of the solutions was measured before use and the concentration of bilirubin in injectates and perfusates was calculated from the known sp act of the [3 H]bilirubin used.

Experimental Procedure. Single-pass studies. The BUI for bilirubin was measured using an adaptation of the method originally described by Oldendorf (7). The injectate was delivered via a cannula placed retrogradely in the external carotid artery as described by Hardebo and Nilsson (8) rather than by direct puncture of the common carotid artery (Fig. 1a). The isotope composition of injectates was 0.4–1.0 μCi [3 H]bilirubin, 0.1–0.25 μCi [14 C] butanol, and 10 μCi ^{99m}Tc as sodium pertechnetate. Butanol was used as a freely diffusible internal reference, almost completely extracted by the brain during a single passage, and sodium pertechnetate, which is nondiffusible, enabled correction for the background intravascular space. The osmolality of the injectates was 160–245 mosmol/kg, and the pH was 7.8–8.3. In the case of free bilirubin uptake studies, an intraperitoneal loading dose of 200 mg/kg of sulfafurazole was given to minimize the effect of any mixing between the injectate and rat plasma.

The injectate was infused as a 200 μL bolus over 1 s and decapitation performed after 5 s. Aliquots of the injectate and brain tissue samples from the frontal, parietal, and occipital lobes of the ipsilateral cerebral hemisphere were prepared for immediate γ counting (Packard Auto-Gamma Spectrometer, model 3003, Packard, Cavesham, Berkshire, England), and, following digestion in soluen 350 (Packard), subjected to double-isotope liquid scintillation counting one wk later (Packard Liquid Scintillation System, Tricarb 2660). The BUI for albumin-bound and free bilirubin was calculated relative to the freely diffusible butanol reference from the respective ratios of tritium to ^{14}C counts in brain and injectate samples. The contribution of residual isotope in the intravascular space represented by sodium pertechnetate was similarly calculated and subtracted from the overall BUI. Equation 1 was used to calculate the BUI for albumin-bound bilirubin. For free bilirubin studies, two further corrections were necessary as described in Results.

$$\text{BUI (\%)} = \frac{\frac{[\text{Bilirubin}]^3}{[\text{Butanol}]^{14}} (\text{brain})}{\frac{[\text{Bilirubin}]^3}{[\text{Butanol}]^{14}} (\text{injectate})} - \frac{\frac{^{99m}\text{Tc}}{[\text{Butanol}]^{14}} (\text{brain})}{\frac{^{99m}\text{Tc}}{[\text{Butanol}]^{14}} (\text{injectate})} \quad (1)$$

In situ brain perfusion. The PA (mL/s/g) of the blood-brain

barrier to bilirubin was determined using the *in situ* brain perfusion technique developed by Takasato *et al.* (9). The isotope composition of perfusates was 0.5 $\mu\text{Ci/mL}$ [^3H]bilirubin, 25 $\mu\text{Ci/mL}$ 99m sodium pertechnetate, and 0.3 $\mu\text{Ci/mL}$ [^{14}C] D-alanine. Sodium pertechnetate served as an intravascular marker and D-alanine was used as a control for blood-brain barrier integrity. The pH of the perfusates was 7.5–8.0. The right common carotid artery was ligated immediately before infusion of the perfusate into the right external carotid artery (Fig. 1b). The rate of infusion was adjusted to provide a pressure greater than the animal's systolic blood pressure, thereby minimizing systemic blood flow to the right cerebral hemisphere. A flow rate of 5 mL/min and a perfusion time of 60 s, terminated by decapitation, were chosen. During the perfusion, systemic blood volume was maintained by withdrawing blood from the femoral artery at the same rate.

Brain tissue samples from the ipsilateral cerebral hemisphere were prepared for γ and liquid scintillation counting as described above for single-pass studies. The cerebrovascular PA for bilirubin and D-alanine were calculated using the equation derived by Takasato *et al.* (9). Equation 2 was used to calculate the PA (mL/s/g) for albumin-bound bilirubin. We used the mean values obtained under the same experimental conditions by Takasato *et al.* (9) for frontal ($13.03 \text{ mL/s/g} \times 10^2$), parietal ($12.76 \text{ mL/s/g} \times 10^2$), and occipital ($11.93 \text{ mL/s/g} \times 10^2$) cerebral perfusion flow. In the case of free bilirubin studies, two further corrections were necessary as described in Results.

$$\text{PA} = -\text{Fpf} \ln_e \left(1 - \frac{\text{C brain (T)}}{\text{T} \cdot \text{Fpf} \cdot \text{Cpf}} \right) \quad (2)$$

Fpf, regional cerebral perfusion fluid flow (mL/s/g); Cpf, tracer concentration in the perfusate (dpm/mL); T, time duration of perfusion (s); and C brain (T), brain parenchymal tracer concentration at decapitation (dpm/g).

Statistics. Statistical significance was estimated by *t* test.

RESULTS

Single-pass studies. The mean arterial blood pressure, pH, and respiratory blood gas tensions were within the physiologic range and comparable in the two study groups (control injectate and injectate + sulfonamide) at the time of the experiments (Table 1).

Centrifugation of injectate samples demonstrated that, after albumin-binding and membrane dialysis, $4.1 \pm 2.4\%$ (mean $\pm$ SD, $n = 14$) of the tritium activity remained unincorporated. One h after addition of sulfadimethoxine to a portion of the injectate solution, the tritium activity in the filtrate increased by a further $16.5 \pm 4.2\%$ (mean $\pm$ SD, $n = 18$). This was attributed to displacement of bilirubin from albumin, and was not found to increase significantly with time over the subsequent 3 h. The total concentration of bilirubin in the albumin-bound control injectates was $493 \pm 157 \text{ nmol/L}$ (mean $\pm$ SD, $n = 14$), and in the sulfadimethoxine-treated injectates was $443 \pm 108 \text{ nmol/L}$ (mean $\pm$ SD, $n = 18$). The concentration of free bilirubin

displaced by the sulfonamide was $68 \pm 28 \text{ nmol/L}$ (mean $\pm$ SD, $n = 18$).

The measured BUI for bilirubin in the control injectates was $1.8 \pm 0.8\%$ (mean $\pm$ SD, $n = 14$). This could be more than accounted for by the percentage of tritium activity shown to be dialyzable *in vitro*, and was therefore attributed to unincorporated tritium in the injectate. Two corrections were therefore made in calculating the uptake of free bilirubin from sulfonamide-treated injectates. First, the corresponding apparent uptake from the control portion of the prepared solution was subtracted. The BUI was then calculated as a percentage of the measured free bilirubin available in individual injectates, *i.e.* the percentage of [^3H]bilirubin demonstrated *in vitro* to be unbound by virtue of being dialyzable. There was no significant difference between the BUI determined in frontal, parietal, and occipital lobes and a mean value was calculated for the cerebral hemisphere in each individual animal. The BUI for free bilirubin was 28.5 ± 9.3 (mean $\pm$ SD, $n = 18$) (Fig. 2).

In situ brain perfusion studies. The mean arterial blood pressure, pH, and respiratory blood gas tensions were within the physiologic range and comparable in the two study groups (control perfusates and perfusates + sulfonamide) at the time of the experiments (Table 1).

Centrifugation of perfusate samples demonstrated that after albumin-binding and membrane dialysis $3.3 \pm 1.1\%$ (mean $\pm$ SD, $n = 13$) of the tritium activity remained unincorporated. One h after addition of sulfadimethoxine to a portion of the perfusate solution, the tritium activity in the filtrate increased by a further $17.3 \pm 6.9\%$ (mean $\pm$ SD, $n = 12$). This was attributed to displacement of bilirubin from albumin. The total concentration of bilirubin in the control perfusates was $67 \pm 19 \text{ nmol/L}$ (mean $\pm$ SD, $n = 13$) and in the sulfadimethoxine-treated perfusates was $65 \pm 18 \text{ nmol/L}$ (mean $\pm$ SD, $n = 12$). The concentration of free bilirubin displaced by the sulfadimethoxine was $11 \pm 4 \text{ nmol/L}$ (mean $\pm$ SD, $n = 12$). The measured PA value for bilirubin in the control perfusates was $1.91 \pm 0.98 \times 10^{-4} \text{ mL/s/g}$ (mean $\pm$ SD, $n = 13$). This could be accounted for by unincorporated tritium in the perfusate. The PA value for free bilirubin in sulfonamide-treated perfusates was calculated using the same corrections as described for the BUI, thus allowing for unincorporated tritium and the percentage of albumin-bound bilirubin shown *in vitro* to have been displaced in individual perfusates. There was no significant difference in the PA values obtained for the frontal, parietal, and occipital lobes, and a mean value was therefore calculated for the cerebral hemisphere as a

Fig. 2. The mean BUI values for control and sulfonamide-treated injectates. Error bars represent 1 SD.

Table 1. Mean arterial blood pressure (MABP), pH, and respiratory blood gas tensions (mean $\pm$ SD) at the time of studies

Study group	n	MABP (mm Hg)	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)
<i>Single pass</i>					
Control injectate	14	136 $\pm$ 17	7.35 $\pm$ 0.03	43 $\pm$ 3	97 $\pm$ 13
Injectate + sulfonamide	18	139 $\pm$ 17	7.34 $\pm$ 0.04	42 $\pm$ 5	95 $\pm$ 9
<i>In situ perfusion</i>					
Control perfusate	13	109 $\pm$ 18	7.35 $\pm$ 0.06	43 $\pm$ 6	101 $\pm$ 16
Perfusate + sulfonamide	12	105 $\pm$ 13	7.35 $\pm$ 0.05	41 $\pm$ 8	98 $\pm$ 16

whole. The cerebrovascular PA value for free bilirubin was $54.84 \pm 36.38 \times 10^{-4}$ mL/s/g (mean $\pm$ SD, $n = 13$) (Fig. 3).

The PA values for D-alanine, added to the control and sulfonamide-treated perfusates, were $5.02 \pm 3.60 \times 10^{-4}$ mL/s/g (mean $\pm$ SD, $n = 13$) and $5.80 \pm 4.06 \times 10^{-4}$ mL/s/g (mean $\pm$ SD, $n = 12$) (NS), respectively. Although there are no other reported values for the PA of D-alanine for comparison, it would be expected to be low as the K_m for L-alanine is $183 \mu\text{mol/mL}$ (10). In contrast to other amino acids for which PA values have been determined, our value for D-alanine is, for example, approximately 20% of that quoted for phenylalanine (11).

DISCUSSION

Difficulties arising from the biochemistry of the bilirubin molecule and the limitations imposed by the experimental techniques used require discussion before interpretation of the results in the context of the newborn infant.

As commercially available radiolabeled bilirubin was of too low a specific activity to meet the requirements of the study, tracer of the appropriate specific activity had to be synthesized. Care is required with regard to ambient lighting and the pH of solutions containing bilirubin in view of bilirubin's chemical instability, ready isomerisation, photosensitivity, and low aqueous solubility. *In vitro*, and particularly at acid pH, the isomers bilirubin III- α and XIII- α are formed by cleavage of bilirubin IX- α at the central methylene bridge with subsequent recombinations of the dipyrrole units. Crystalline bilirubin (Sigma Chemical Co., St. Louis, MO, cat. no. B4126) contains approximately 1–1.5% of the III- α and 5–7% of the XIII- α isomers. These isomers accumulated further during the synthesis of [^3H]bilirubin, comprising approximately 15% of the product, and were removed by thin-layer chromatography. On retrieval of [^3H]bilirubin from storage vials and during preparation of the injectate/perfusate solutions, subdued lighting was used and an alkaline pH (> 9.0) was maintained until adjustment to pH 7.5–8.3 before purification and use. The high pH used was a necessary precaution in view of the low aqueous solubility of bilirubin in the physiologic range. At pH 7.4, the solubility of bilirubin dianion is 7 nmol/L (2). The radiochemical purity of stored [^3H]bilirubin was documented to the limits of

the techniques available. The proportion of activity that remained dialyzable in the presence of a 20-fold M excess of albumin, which would effectively bind all bilirubin, was assumed to be unincorporated tritium, and attempts were made to remove this by prolonged membrane dialysis. Membrane filtration cones with a retention cutoff of 25 000 mol wt were chosen to assess the efficiency of this purification process and the displacing effect of the sulfonamide. They were used at low relative centrifugal force to avoid or minimize protein leakage.

The small percentage of apparently unincorporated tritium activity quantified in the control injectates and perfusates more than accounted for the uptake and permeability values observed in the control (albumin-bound bilirubin) experiments. The need for membrane dialysis to purify [^3H]bilirubin immediately before use dictated the addition of a displacing agent to the injectate/perfusate to provide free bilirubin. Any direct effect of the sulfonamide on blood-brain barrier permeability was excluded in the *in situ* brain perfusion studies by demonstration of the constancy of the D[U- ^{14}C]alanine PA product in the control and sulfonamide-treated groups. D-alanine was used as a non-transported substance of a mol wt and low lipid solubility comparable to bilirubin.

After Oldendorf's original description of single-pass carotid bolus studies in the rat (7), the technique was modified. The traditional [^3H]water reference was replaced by more diffusible substances, and the study duration of 15 s reduced. Butanol was used in these studies since it is largely extracted into brain tissue within a single capillary transit time (less than 1 s) and leaves the brain slowly at a rate of 1.1%/s (12). To limit this efflux, which will tend to overestimate the BUI of the test substance, the time to decapitation was reduced to 5 s. A further advantage of reducing the study time is to minimize the effects of recirculation of tracer that occurs after 7–8 s (8). Some 96% of the injectate solution clears the brain by 2 s, and there is very little remaining at 5 s (8). Correction for residual tracer in the vascular space was made in these experiments by inclusion of nondiffusible $^{99\text{m}}\text{Tc}$. A further important consideration when using single-pass techniques to study the uptake of albumin-bound substances is the degree of mixing that may occur between the injected bolus and circulating rat plasma. Pardridge *et al.* (13) have documented that the upper limit for bolus mixing is 5.5%. To minimize the effect of bolus mixing in the free bilirubin uptake studies, an intraperitoneal loading dose of 200 mg/kg sulfafurazole was given.

The *in situ* brain perfusion technique used in the second series of experiments provides a quantitative measure of blood-brain barrier permeability. Initial studies using the *in situ* technique (9) endeavoured to maintain normal cerebral function and metabolism by using oxygenated perfusates containing glucose. Such precautions are unnecessary for short duration perfusions of one hemisphere. Greenwood *et al.* (14) have demonstrated maintenance of blood-brain barrier integrity for up to 30 min during *in situ* perfusion of the whole brain with a solution containing the metabolic inhibitor 2,4-dinitrophenol.

The single-pass and *in situ* perfusion experiments both demonstrate a significant permeability of the microvasculature of the adult rat cerebral hemisphere to unbound bilirubin. The methodology is unsuitable for studying areas of the brain such as the basal ganglia and cerebellum, but regional values were obtained from the frontal, parietal, and occipital lobes. In neither set of experiments was there any significant difference between the uptakes of these three brain regions. The BUI observed for free bilirubin of $28.5 \pm 9.3\%$ is considerably greater than that reported for aqueous soluble polar compounds such as mannitol, but very much less than that for highly lipid-soluble compounds such as propranolol and caffeine, which attain BUI in excess of 70%. Similarly, the PA value for free bilirubin of $54.84 \pm 36.38 \times 10^{-4}$ mL/s/g indicates that the permeability of the rat blood-brain barrier to free bilirubin is significant because it approaches that of molecules such as antipyrine with a PA in the region of

Fig. 3. The mean PA values for control and sulfonamide-treated perfusates. Error bars represent 1 SD.

140×10^{-4} mL/s/g (9). Both the BUI and PA product are far greater than would be expected for a molecule that exhibits such low lipid solubility *in vitro* (2).

Albumin-stripping (the dissociation of bilirubin from albumin during brain capillary transit) is unlikely to have contributed significantly to these results. The capillary transit time in the rat is about 1 s, and applying a dissociation rate constant for bilirubin of 8.8×10^{-3} /s (15) would provide 0.88% of stripped bilirubin available for uptake. Assuming the sulfonamide does not affect the dissociation rate constant significantly, such a value would be masked by the results for BUI and PA of free bilirubin but may have contributed to the apparent control values. Two other variables that may have had a greater influence on the results are those of pH and temperature. The pH will influence the relative proportions of bilirubin subspecies, namely the dianion, acid-salt, and acid. At the pH range of the injectates/perfusates (7.5–8.3), bilirubin dianion would predominate, and the acid-salt and acid, which are the subspecies implicated in membrane binding, would be in very low concentration. Although the binding of bilirubin to albumin is independent of pH within the physiologic range, the binding of bilirubin to cell membranes is enhanced at lower pH (16). Despite these considerations, no significant correlation could be demonstrated between the pH of the solutions and PA or BUI values. The injectate and perfusate solutions were not buffered and may have been altered in the direction of more physiologic pH values during transit.

Temperature significantly influences bilirubin binding to albumin. Ideally, all manipulations of the solutions, including centrifugation and administration, would have been performed at 37°C. Consideration of the potential for increased degradation of [3 H]bilirubin at this temperature prompted the use of room temperature throughout. In the case of the single-pass studies, it is likely that the injectate bolus temperature would tend to equilibrate with body temperature during passage. This would be unlikely to occur with the *in situ* perfusions over 60 s, during which right cerebral hemisphere cooling may have occurred. If the temperature of the injectate in single-pass studies had been raised to 37°C during passage, the free bilirubin content would theoretically have increased by up to 3-fold (17). The BUI value, based on the *in vitro* measurement of free bilirubin at room temperature, could as a result be an overestimate by the same factor. Overestimation of both the BUI and PA values may also have resulted from binding of free bilirubin to the membrane cones during centrifugation. Bilirubin binding to membranes such as Sephadex is known to occur at lower pH (18), but the use of solutions with a pH ≥ 7.5 may have limited this potential error in our experiments.

There are several constraints to direct extrapolation of these results to the mode of entry of bilirubin in the human newborn brain. The differences among species, developmental age, and region of the brain studied all merit comment. The rat was used because both experimental techniques have been developed and validated in that species. Rat albumin, however, differs in its binding characteristics with bilirubin; for this reason human albumin was used in the preparation of injectates and perfusates. Technical consideration dictated the use of adult rats. Animal studies of the newborn brain (19) have demonstrated a longer capillary transit time, which, if it exceeds the dissociation time of an albumin-bound compound, will increase brain uptake. Such albumin-stripping has been demonstrated in the case of the protein-bound anticonvulsants phenobarbitone and phenytoin in newborn as opposed to adult rabbits (19), but, as previously discussed, would not contribute significantly in the case of bilirubin, which is more tightly bound to albumin. Conversely, the increased blood flow to subcortical regions of the brain has been proposed as the mechanism of increased bilirubin deposition in those areas (20). A comparative study of areas such as the basal ganglia and cerebellum was not feasible using the techniques described.

The results do, however, demonstrate that despite the evidence

of bilirubin's very low lipid solubility *in vitro* (2), its behavior *in vivo* in relation to the cerebral microvasculature corresponds to that of a lipid soluble molecule. This may be the result of an interaction between bilirubin and membrane phospholipids such that lipid soluble complexes of bilirubin are formed (2). Alternatively, Wennberg (21) has recently suggested that the tissue binding of bilirubin results from detergent properties of the free bilirubin acid-salt.

The use of [3 H]bilirubin IX- α of high sp act in these experiments allowed the study of blood-brain barrier permeability to unbound bilirubin at concentrations known to exist in newborn infants. Although the effect of exposure of the brain to such levels for longer duration was not addressed by this study, estimation of the rate of influx of free bilirubin using these results provides values well below the rate at which brain bilirubin oxidase would be expected to metabolize the molecule (22). This would suggest that contributory factors may be necessary to confer neurotoxicity. For instance, the metabolism of bilirubin by brain mitochondrial bilirubin oxidase may be insufficient under conditions of hypoxia and therefore allow the pigment to accumulate to toxic levels. This mechanism of bilirubin neurotoxicity has been proposed by Brodersen (23) and may explain the combined effect of hypoxia and hyperbilirubinemia in disrupting brain energy metabolism in guinea pig cerebral hemisphere slices studied *in vitro* (24). Hypoxia in common with asphyxia, hypercapnia, hypertension, and hyperosmolality may also exacerbate bilirubin encephalopathy by disruption of the blood-brain barrier. The resultant increase of influx of bilirubin may overwhelm the clearance capacity of brain bilirubin oxidase, and allow bilirubin to attain concentrations that promote precipitation of bilirubin acid on intracellular organelles. Such acute effects of blood-brain barrier opening in the presence of hyperbilirubinemia have been demonstrated in a rat model of hyperosmolar opening (25), both in terms of electrophysiologic (26) and 31 P nuclear magnetic resonance spectrographic (27) evidence of cerebral neurotoxicity.

In conclusion, these results provide the first quantitation of blood-brain barrier permeability to bilirubin, and demonstrate significant permeability to free, but not albumin-bound, bilirubin.

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