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Research Article

Experimental Bilirubin Encephalopathy. The Mode of Entry of Bilirubin-^{14}C into the Central Nervous System *

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Bilirubin encephalopathy (kernicterus) is a neurologic complication of unconjugated hyperbilirubinemia confined almost entirely to newborn infants (2–4). A similar neurologic disorder is observed in a mutant strain of Wistar rats (Gunn) with hereditary unconjugated hyperbilirubinemia (5, 6). In this type of disturbance the brain usually shows localized deposits of yellow pigment, which has been identified in part as unconjugated bilirubin (7, 8). Although these observations do not provide direct proof that unconjugated bilirubin is responsible for the neuropathologic changes characteristic of kernicterus, experiments *in vitro* have left little doubt that this bile pigment interferes with vital cellular functions (9–11). The precise nature of this toxicity is unknown, but interference with oxidative phosphorylation in mitochondria has been suggested as a possible mechanism (12–14), and recent findings in icteric rats indicate that the most intensely pigmented regions of the brain show reduced ATP concentrations (15).

Most of the bilirubin in the plasma is bound firmly to albumin (16), but several observations make it appear doubtful that bound pigment enters the brain. In general, the blood-brain barrier would be expected to be relatively impermeable for water soluble molecules as large as albumin (17, 18). Indeed, in studies with labeled albumin

virtually all of the small amount of isotope detected in the central nervous system appeared to be confined to the vascular space (19–21). Moreover, *in vitro*, the toxicity of bilirubin for cellular respiration was abolished by binding of the pigment to albumin (14). These observations are consistent with the supposition that only unbound bilirubin crosses the blood-brain barrier and gains access to the central nervous system (6, 22, 23), although this concept is not universally accepted (24, 25).

At pigment concentrations not exceeding the total binding capacity of the albumin, it is apparent that the fraction of unbound plasma bilirubin available for transport into the brain is very small (16, 22). Human albumin has been found to bind the pigment more firmly than rat or guinea pig albumin (26), but to date methodologic difficulties have prevented the precise measurement or calculation of the individual binding constants (16, 22). It also has been demonstrated that several organic anions may competitively displace bilirubin from its albumin-binding sites and thereby increase the unbound pigment fraction *in vitro* (22, 27). In Gunn rats, infusion of these compounds results in a sharp decline of the plasma bilirubin concentration and a concomitant escape of unbound pigment from the vascular compartment (6, 26). Furthermore, because of its higher binding affinity, human albumin infused into such animals is capable of returning bilirubin from the tissues to the circulation (26).

This information was utilized to determine whether bilirubin deposition in the brain is influenced by alterations in the pigment-binding properties of the plasma. Conclusive evidence was obtained that only unbound bilirubin is able to cross the blood-brain barrier, and that the pigment level in the nervous system and bilirubin neurotoxicity are related to the unbound, rather than to the total, pigment concentration in the plasma (1).

Methods

Preparation of experimental animals. All experiments were carried out on newborn guinea pigs and adult Gunn rats (28). These animals are unable to conjugate (29, 30) and excrete (31) infused bilirubin-^{14}C so that reasonably constant and predictable pigment levels could be maintained for the duration of the experiments. Pregnant guinea pigs within an estimated 2 weeks of delivery were anesthetized with ether and the fetuses, weighing from 60 to 120 g, delivered by Caesarian section. Animals exhibiting respiratory distress or poor motility were discarded.

A PE-10 polyethylene catheter [1] for continuous infusion was inserted in a superficial vein along the antero-lateral aspect of an upper foreleg. The animals were anesthetized with ether for less than 15 minutes, and then restrained for the remainder of the experiments. Hypoglycemia and dehydration were prevented by subcutaneous administration of 2 ml isotonic glucose per 100 g body weight. At the conclusion of the experiments, 1 to 2 ml of blood was obtained by cardiac puncture or from the neck after decapitation. If samples were required during the experiments, 0.2 ml of blood was aspirated from the exposed jugular vein opposite the side of infusion.

In adult Gunn rats weighing from 188 to 372 g, a PE-10 polyethylene catheter was introduced into a femoral vein, and the animals were allowed to awaken from the ether anesthesia in a restraining cage. Patency of the catheter was maintained by slow infusion of isotonic saline. Serial blood samples were collected from the tip of the tail (30). At the conclusion of the experiments blood was obtained by cardiac puncture, and the rats were decapitated.

Preparation and infusion of pigment-containing solutions. Unconjugated crystalline bilirubin-^{14}C (32) with a specific activity of 7,000 to 100,000 dpm per μmole was dissolved in 0.1 N NaOH in the dark. The pigment was rapidly mixed with an aqueous solution of human albumin,[2] adult guinea pig serum, or sodium taurocholate in isotonic saline. The pH was adjusted to 8 with 1 N acetic acid. The final composition per 100 ml of the three pigment solutions was as follows: 250 mg bilirubin-^{14}C bound to 20 g human albumin; 500 mg bilirubin-^{14}C dissolved in three parts guinea pig serum and one part isotonic saline, the total solution containing 2.7 g guinea pig albumin; and 500 mg bilirubin-^{14}C dissolved in 5 mM sodium taurocholate in isotonic saline. In a few experiments, the bilirubin-^{14}C human albumin solution also contained 5 mM sodium taurocholate.

Conjugated bilirubin-^{14}C was prepared by infusing bilirubin-^{14}C into an adult Sprague-Dawley rat with an external bile fistula (33). The bile collected at 4° C in the dark contained 114 mg per 100 ml of conjugated bilirubin-^{14}C that exhibited a SA of 180,000 dpm per μmole.

[1] Clay-Adams, Inc., New York, N. Y.
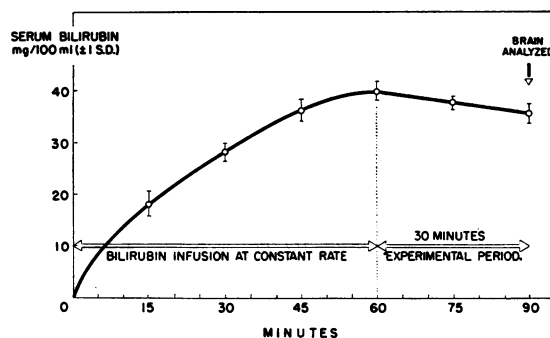
[2] Albumisol, 25%, Merck Sharp and Dohme, West Point, Pa.

FIG. 1. SERUM BILIRUBIN LEVELS IN 16 NEWBORN GUINEA PIGS INFUSED FOR 1 HOUR WITH BILIRUBIN-^{14}C BOUND TO HUMAN ALBUMIN. The constant infusion rate was 50 μg per g body weight per hour. This design served as a prototype for all experiments. In the 30-minute period after infusion, serum bilirubin levels declined in a predictable fashion, unless the pigment-binding properties of the plasma were altered by additional experimental manipulations. Radioactivity in the brain was determined at the end of this "experimental period."

These radioactive pigment solutions were infused into the newborn guinea pigs at a constant rate for 1 hour.[3] The infusion rates were determined empirically so that the serum bilirubin concentration at the end of the infusion was 30 to 40 mg per 100 ml. All pigment-containing solutions were protected from light during the infusions, and the specific activity of the bilirubin remained unchanged. A prototype of the experimental design is given in Figure 1. In the 30-minute period after infusion, serum bilirubin levels declined in a predictable fashion except when the pigment-binding properties of the plasma were altered by additional experimental manipulation.

In adult Gunn rats the experimental procedure was similar, regardless of initial serum bilirubin concentrations.

Analytical procedures. At the end of the experimental period, the animals were decapitated, and the brain was removed. Adherent blood, meninges, and the choroid plexus were carefully wiped off with cotton swabs. The brain was divided by a section of the midsagittal plane, and each half was homogenized separately for 2 minutes with 1 vol per weight glacial acetic acid and 3 vol per weight chloroform in a Potter-Elvehjem tissue grinder. The chloroform phase was separated and the aqueous residue re-extracted with 3 vol chloroform. The combined chloroform phases were evaporated in low potassium counting vials, and the residue was dissolved in 3 ml of 0.3 M Hyamine in methanol and 17 ml of scintillator solution for radioassay (33). Bilirubin concentration in each half of the brain was calculated by dividing radioactivity per g tissue (corrected for label present in the vascular space, as described below) by the specific ac-

[3] Infusion-withdrawal pump, model 600–930 V, Harvard Apparatus Co., Dover, Mass.

tivity of the serum bilirubin at death. In all experimental groups, a one-sample t test was performed on the difference between the right and left sides of each brain (34). Statistically significant differences were not found.

Six recovery experiments with this extraction procedure, performed with brains to which known amounts of bilirubin-^{14}C had been added, yielded 88.7% (SD ± 1.4) of the added radioactivity. In two instances when brains containing endogenous bilirubin-^{14}C were homogenized with added unlabeled carrier bilirubin, crystallization and radioassay of the extracted pigment (32) indicated that 96% of the recovered isotope in the brain was present in bilirubin.

Brain of three newborn guinea pigs infused with conjugated bilirubin-^{14}C was homogenized with 4 vol of 0.5 M Hyamine in methanol. For radioassay, 2 ml of the homogenate was mixed with the scintillator fluid and sufficient methanol to prevent separation of the mixture into two phases. Recovery experiments with this technique, carried out by homogenizing known amounts of conjugated bilirubin-^{14}C with fetal guinea pig brain, yielded 93% of the added radioactivity (32).

The volume of the plasma compartment in the brain of jaundiced animals was determined by injection of a tracer dose of ^{131}I-labeled human albumin[4] or ^{125}I-labeled guinea pig albumin[5] (35). In 12 newborn guinea pigs infused with bilirubin-containing solutions and kept in humidified room air for 30 minutes, the plasma compartment was 0.64 ml (SD ± 0.07) per 100 g brain wet weight. In six animals treated with salicylate or hydrochloric acid after the infusion of albumin-bound bilirubin, the plasma compartment was 0.76 ml (SD ± 0.04) per 100 g brain wet weight. After exposure of two icteric guinea pigs for 30 minutes to 25% CO_2 in oxygen, the corresponding figures were 0.88 and 0.92 ml. The plasma compartment of the brain of three Gunn rats was 0.75 (0.71 to 0.79) ml per 100 g brain wet weight. These values were used to correct the total radioactivity extracted from the brain for radioactive pigment contained in the vascular compartment.

In two newborn guinea pigs and four adult Gunn rats infused with bilirubin-^{14}C in taurocholate, the brain was dissected into five distinct anatomical areas to permit regional analysis of radioactivity in the cortex, basal ganglia and hypothalamus, epithalamus and midbrain, cerebellum, and brain stem. The size of the plasma compartment in these areas was determined in three other animals and was found to range from 0.8 to 1.27 ml per 100 g wet weight, the cerebellum giving the highest value.

Serum bilirubin was measured by the diazo reaction (36) and its specific activity determined as described (32). The hematocrit was estimated by a microprocedure. Total serum protein concentration was measured with the biuret method (37), and the albumin fraction was

calculated after electrophoresis on cellulose acetate strips [6] (38). Human and guinea pig albumin were separated by electrophoresis and determined individually (39). Serum salicylate levels were estimated colorimetrically (40), and whole blood pH was measured at 37° C.[7]

All samples containing ^{14}C activity were assayed in a Packard Tri-Carb liquid scintillation spectrometer, and radioactivity was expressed in disintegrations per minute (30, 33). Samples containing ^{131}I- or ^{125}I-labeled albumin were assayed in a well-type gamma scintillation spectrometer,[8] and radioactivity was expressed in counts per minute.

Ancillary studies. Total plasma volume was determined with human albumin-^{131}I in three untreated newborn guinea pigs and in 19 animals infused with pigment-containing solutions (35). Total serum protein and albumin concentrations were determined in nine animals before infusion of the solutions and at the conclusion of the experiments.

Because albumin from different species has been shown to differ in the degree of pigment binding (26), the relative affinity of this protein fraction for bilirubin-^{14}C was studied in guinea pig serum that contained various amounts of human albumin. Twenty-seven μg of bilirubin-^{14}C (SA 10,000,000 dpm per μmole) was dissolved in 0.05 ml 0.1 N NaOH in the dark and mixed immediately with 0.95 ml of guinea pig serum. The serum contained, per 100 ml, either 1.8 g endogenous guinea pig albumin (37, 38) or 1.4 g endogenous guinea pig albumin and 1.1, 2.3, 3.4, 4.5, or 5.7 g added human albumin. Electrophoresis was carried out on cellulose acetate with a modified Owen buffer (41). Narrow rims on either side of the electrophoretic strip were stained with Ponceau S dye (42) for identification of the various protein fractions. Accordingly, it was possible to divide the unstained center of the strip into four zones, corresponding to human albumin, guinea pig albumin, globulins, and supporting medium without protein. The cellulose acetate of each zone was added to counting vials for radioassay in a liquid scintillation spectrometer, and the radioactivity of the individual protein fractions was expressed as a percentage of the total amount of isotope recovered from the electrophoretic strip.

Studies of bilirubin neurotoxicity in vivo. In a first series of experiments, an attempt was made to correlate the anticipated bilirubin concentration in the brain with neurologic function and viability of the animals. Forty-five newborn guinea pigs were infused for 1 hour with bilirubin either bound to human albumin or dissolved in guinea pig serum. An additional four control animals received guinea pig serum without the pigment. Immediately after the infusions, the young guinea pigs were returned to their mothers or to "wet nurses." The ani-

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[4] 20 μc per ml, E. R. Squibb and Sons, New Brunswick, N. J.

[5] 363 μc per ml, E. R. Squibb and Sons.

[6] Consolidated Laboratories, Inc., Chicago Heights, Ill.

[7] Model 76 expanded scale pH meter with micro blood pH assembly, Beckman Instruments, Inc., Fullerton, Calif.

[8] Instrument and Development Products Co., Chicago, Ill.

mals were periodically examined for neurologic manifestations such as opisthotonus, impaired righting reactions, ataxia, and head tremor, and the length of survival of affected animals was recorded. Guinea pigs that appeared neurologically normal were killed after 24 hours. At death, serum bilirubin concentration was determined, and the brain was removed and fixed in buffered formalin for gross and histologic examination.

In a second series of experiments, an attempt was made to evaluate neurologic function and viability in animals in which bilirubin had been deposited in the brain and subsequently removed by infusion of human albumin. Twenty-five spontaneously delivered newborn guinea pigs were infused for 1 hour at a constant rate with bilirubin dissolved in guinea pig serum. The animals were then divided into two groups; one was treated with 25 g per 100 ml human albumin solution, the other with isotonic saline. These infusions lasted 30 minutes at a constant rate of 0.06 ml per g per hour. The newborn guinea pigs were then returned to their mothers, observed for neurologic damage, and the survivors killed after 24 hours. Serum bilirubin concentration was measured at death and the brain examined for yellow pigmentation.

Results

Experiments in newborn guinea pigs. Nine animals were infused with bilirubin-^{14}C bound to human albumin at a rate of 50 μg per g body weight per hour. The molar ratio of bilirubin to albumin in the infusate was approximately 1.5 to 1.0, which is within the binding capacity of this protein fraction (16, 26). The infusions increased the average plasma volume from 5.4 ml to 8.7 ml per 100 g (Table I). At the conclusion of the experiments, most of the infused human albumin (Table I) and approximately two-thirds of the administered bilirubin-^{14}C (Figures 1, 2) were still in the circulation, yielding a molar pigment to human albumin ratio of 1. The average bilirubin concentration in the serum was 36 mg per 100 ml, whereas
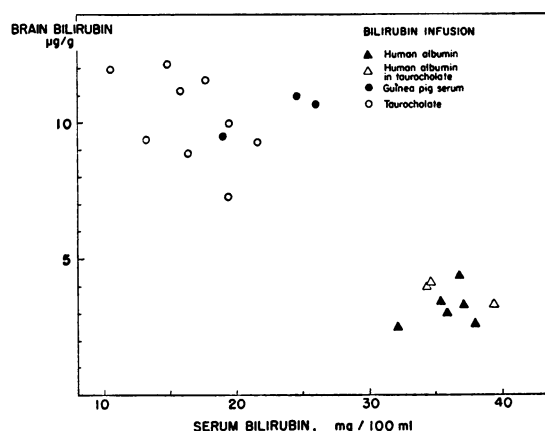

FIG. 2. BRAIN AND SERUM BILIRUBIN LEVELS IN NEW-BORN GUINEA PIGS INFUSED WITH SOLUTIONS CONTAINING BILIRUBIN-^{14}C. The infusions consisted of pigment bound to human albumin, or dissolved in guinea pig serum or taurocholate. During the experimental period, the animals were kept in humidified room air.

the brain contained only 3.4 μg per g (Figure 2). Addition of 5 mM sodium taurocholate to the infusate did not affect the pigment distribution between plasma and brain (Figure 2).

Twelve newborn guinea pigs were infused with bilirubin-^{14}C dissolved in guinea pig serum or taurocholate solution at a rate of 100 μg per g body weight per hour. The administered pigment greatly exceeded the total bilirubin binding capacity of the circulating albumin, including the infused protein (26) (Table I). Consequently, a major fraction of the infused pigment was lost from the circulation into the tissues. The brain contained an average of 10.3 μg of bilirubin per g, whereas the serum concentration was only 18.2 mg per 100 ml (Figure 2). Thus, the ratio between bilirubin concentration in the brain and

TABLE I

Plasma volume and serum proteins in untreated and infused newborn guinea pigs

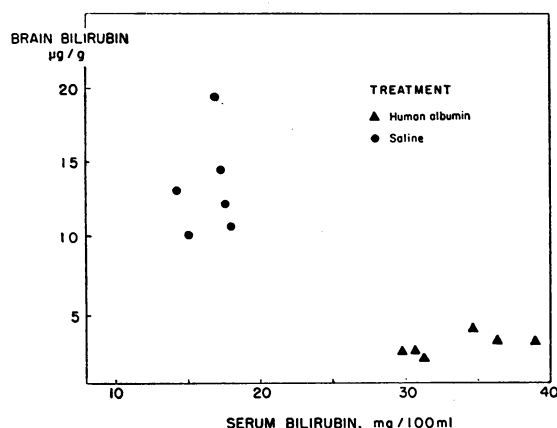|	Plasma volume			Total protein			Guinea pig albumin		Human albumin	
	No. of animals	Mean	Range	No. of animals	Mean	Range	Mean	Range	Mean	Range
		ml/100 g			*g/100 ml*		*g/100 ml*		*g/100 ml*	
Untreated	3	5.4	5.3– 5.6	9	5.4	4.8–6.0	3.6	3.1–4.0		
Infused with bilirubin bound to human albumin	10	8.7	7.2–10.3	6	6.9	6.1–8.4	1.6	1.4–1.9	4.5	4.0–5.4
Infused with bilirubin in taurocholate	9	6.4	5.2– 7.9	3	5.2	5.0–5.3	3.3	3.2–3.5		

FIG. 3. BRAIN AND SERUM BILIRUBIN LEVELS IN NEW-BORN GUINEA PIGS INFUSED WITH BILIRUBIN-^{14}C IN TAURO-CHOLATE AND SUBSEQUENTLY TREATED WITH EITHER HUMAN ALBUMIN OR SALINE.

serum was six times higher in these animals than in those infused with pigment bound to human albumin.

This difference was further illustrated by an experiment in which 12 newborn guinea pigs were infused with bilirubin-^{14}C dissolved in taurocholate solution at a rate of 100 μg per g body weight per hour. During the subsequent experimental period, the animals were kept in room air and infused with either isotonic saline or 25 g per 100 ml human albumin at a constant rate of 0.04 ml per g body weight per hour. In the animals given saline, the brain contained 13.3 μg of bilirubin per g and the serum 16.5 mg per 100 ml (Figure 3). With human albumin, on the other hand, much of the pigment deposited in the brain was mobilized and returned to the circulation, resulting in a fall of brain bilirubin concentration to 2.6 μg per g, whereas the serum concentration rose to 33.7 mg per 100 ml. In these two groups of guinea pigs, the ratio between the pigment concentration in the brain and serum differed approximately tenfold.

A group of six animals was infused with bilirubin-^{14}C bound to human albumin at a rate of 50 μg per g body weight per hour. During the experimental period, treatment with 0.156 M sodium salicylate at a rate of 0.04 ml per g body weight per hour resulted in a marked pigment shift out of the plasma (Figure 4). This organic anion has been shown to compete with the pigment for common binding sites on the protein (22, 26).

The circulating salicylate level was 32.6 (SD ± 1.24) mg per 100 ml serum, and in the two animals in which it was determined, the blood pH was 7.37 and 7.36. Under these conditions, the brain contained 16.5 μg of bilirubin per g, and the serum level was 22.0 mg per 100 ml (Figure 4). By contrast, in six animals prepared identically but treated with saline instead of salicylate, the bilirubin concentration in the brain was only 4.4 μg per g, whereas the corresponding serum level was 34.9 mg per 100 ml (Figure 4). Thus, in guinea pigs infused with the same amounts of bilirubin and human albumin, salicylate increased sixfold the brain to serum bilirubin ratio.

The effect of pH on the ratio between pigment concentrations in the brain and serum was studied in guinea pigs with induced respiratory or metabolic acidosis. Six animals infused at the rate of 50 μg per g body weight per hour with bilirubin-^{14}C bound to human albumin were exposed during the experimental period to 25% CO_2 in oxygen, which resulted in a blood pH of 7.09 to 7.10. This was associated with a fall in the average serum bilirubin level from 36 mg to 26.3 mg per 100 ml and a concomitant increase in the brain pigment concentration from 3.4 μg to 7.9 μg per g (Figure 5). Exposure to 25% CO_2, therefore, increased threefold the brain to serum bilirubin ratio. In three additional guinea pigs, exposed to only 5% CO_2 in oxygen, the serum pigment level decreased to 29.3 mg per 100 ml, but a concurrent increase in
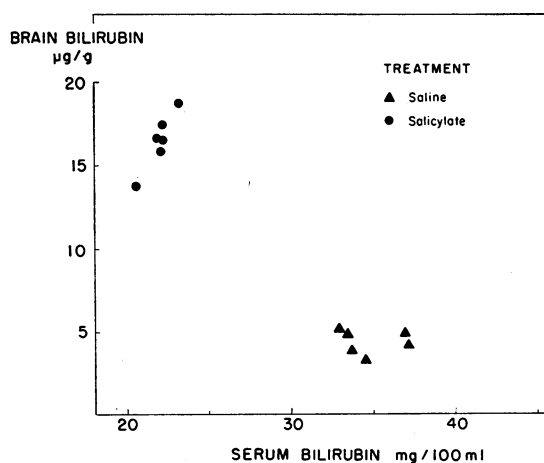

FIG. 4. BRAIN AND SERUM BILIRUBIN LEVELS IN NEW-BORN GUINEA PIGS INFUSED WITH BILIRUBIN-^{14}C BOUND TO HUMAN ALBUMIN AND SUBSEQUENTLY TREATED WITH EITHER SALINE OR SALICYLATE.

the brain could not be demonstrated (Figure 5). In the latter animals, a fall in blood pH was not observed; it ranged from 7.27 to 7.30, as did the pH in the six control guinea pigs exposed to room air.

Metabolic acidosis was induced in six animals by infusion during the experimental period of 0.5 N HCl in saline at a constant rate of 0.04 ml per g body weight per hour. This led to a fall in blood pH to 7.02 (SD ± 0.05) with a concomitant increase of brain bilirubin concentration from 4.4 to 8.6 μg per g (Figure 6). The ratio between pigment concentrations in the brain and serum rose by approximately 2.5, as compared to six control animals infused with saline (Figure 6).
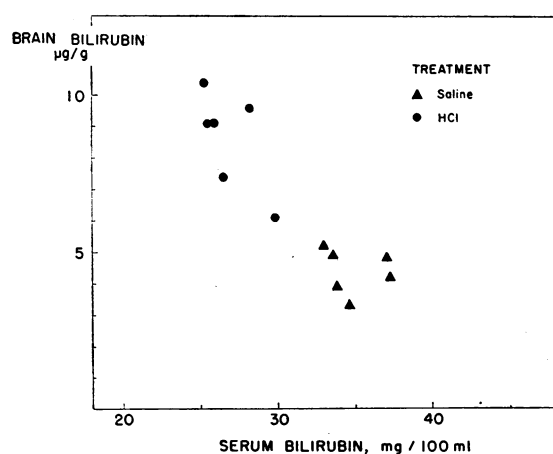

FIG. 5. BRAIN AND SERUM BILIRUBIN LEVELS IN NEW-BORN GUINEA PIGS INFUSED WITH BILIRUBIN-^{14}C BOUND TO HUMAN ALBUMIN AND SUBSEQUENTLY EXPOSED TO EITHER HUMIDIFIED ROOM AIR, 5% CO_2, or 25% CO_2.

Thus, in guinea pigs infused with identical amounts of bilirubin and human albumin, acidosis was associated with an increased bilirubin concentration in the brain and a decline in serum level.

In two newborn guinea pigs infused with bilirubin-^{14}C dissolved in taurocholate solution at a rate of 100 μg per g body weight per hour, separate analysis of the cortex, basal ganglia and hypothalamus, epithalamus and midbrain, cerebellum, and brain stem failed to demonstrate selective accumulation of pigment.

As expected from earlier observations (2, 7, 11, 33, 43), conjugated bilirubin did not enter the central nervous system. In three guinea pigs infused with conjugated bilirubin-^{14}C at a rate of 50
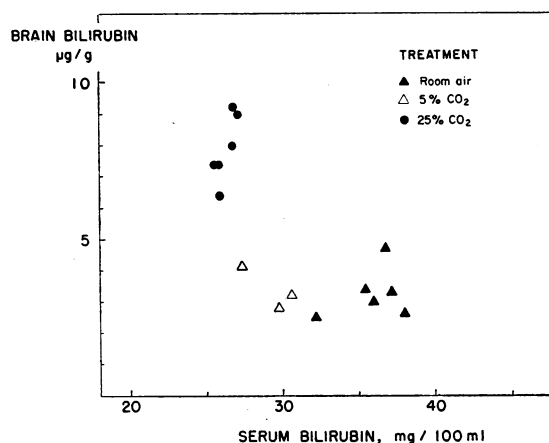

FIG. 6. BRAIN AND SERUM BILIRUBIN LEVELS IN NEW-BORN GUINEA PIGS INFUSED WITH BILIRUBIN-^{14}C BOUND TO HUMAN ALBUMIN AND SUBSEQUENTLY TREATED WITH EITHER HCL OR SALINE.

μg per g body weight per hour, serum levels ranged from 22.5 to 23.5 mg per 100 ml, but significant radioactivity could not be detected in the brain.

Experiments in adult Gunn rats. Experiments similar to those in newborn guinea pigs were carried out in adult Gunn rats. Five rats were infused with labeled bilirubin dissolved in taurocholate and three with the pigment bound to human albumin containing 5 mM taurocholate. An additional three rats were treated with salicylate after the infusion of human albumin-bound bili-
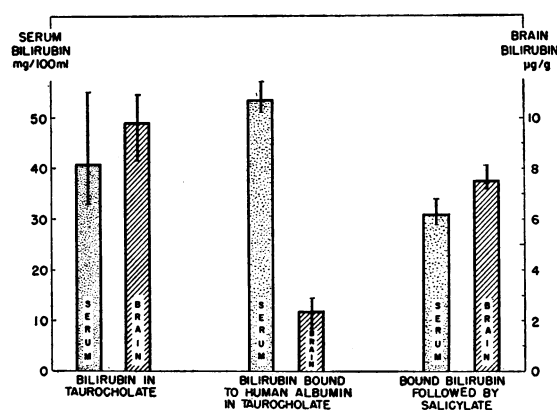

FIG. 7. SERUM AND BRAIN BILIRUBIN LEVELS (AVERAGE AND RANGE) IN ADULT GUNN RATS INFUSED WITH SOLUTIONS CONTAINING BILIRUBIN-^{14}C. The infusions consisted of pigment dissolved in taurocholate, bound to human albumin in taurocholate, or bound to human albumin followed by salicylate.

rubin. All infusion rates were as described for the newborn guinea pigs.

As shown in Figure 7, the results were similar to those obtained in the newborn guinea pigs. Administration of unbound pigment resulted in a five times larger ratio of brain to serum bilirubin concentration, compared with animals infused with albumin-bound material. Moreover, treatment with salicylate after infusion of bound pigment yielded findings similar to those obtained with unbound bilirubin.

In four Gunn rats infused with bilirubin-^{14}C dissolved in taurocholate, separate regional analysis of the central nervous system yielded pigment concentrations ranging from 5.5 to 108.9 μg per g, with the highest values found in the cerebellum and brain stem.

Electrophoretic studies. In agreement with earlier observations made by a different experimental approach (26), human albumin was found to have a stronger binding affinity for bilirubin than guinea pig albumin. In the electrophoretic studies of guinea pig serum containing human albumin, the concentrations of the two albumin fractions were adjusted to reflect the conditions observed in animals infused with human albumin *in vivo* (Table I). Although a small and relatively constant percentage of the applied radioactivity always remained on the supporting medium, including the globulin zone, most of the pigment migrated in the albumin zones (Table II). Of the two albumin fractions, the human protein, regardless of concentration, contained a much larger percentage of the labeled bilirubin than did guinea pig albumin.

TABLE II

Electrophoresis of bilirubin-^{14}C in guinea pig serum enriched with human albumin

Protein in solution			Distribution of radioactivity	
Total protein	Guinea pig albumin	Human albumin	Guinea pig albumin	Human albumin
g/100 ml			*% of total dpm*	
3.9	1.8		76	
4.2	1.4	1.1	22	53
5.4	1.4	2.3	8	67
6.5	1.4	3.4	6	75
7.6	1.4	4.5	10	68
8.8	1.4	5.7	8	70

TABLE III

Studies of bilirubin neurotoxicity in newborn guinea pigs

	Serum bilirubin at death		Number of animals	
	Mean	Range	Alive and active	Moribund or dead
	mg/100 ml			
Guinea pig serum without bilirubin	Trace		4	0
Bilirubin in guinea pig serum	5.9	0.9–12.7	19	11 (7 dead)
Bilirubin bound to human albumin	9.3	3.5–15.3	15	0
Bilirubin in guinea pig serum followed by:				
Saline	10.7	8.5–12.2	3	9 (9 dead)
Human albumin	24.1	14.2–31.2	10	3 (3 dead)

Studies of bilirubin neurotoxicity in vivo. When 30 newborn guinea pigs were infused with bilirubin dissolved in guinea pig serum at a rate of 100 μg per g body weight per hour, 11 developed severe neurologic disturbances and within 24 hours appeared moribund or died (Table III). All four control animals infused with pigment-free serum survived and remained well. Similarly, all 15 guinea pigs treated with bilirubin bound to human albumin infused at a rate of 50 μg per g body weight per hour survived without apparent neurologic damage, although their average serum bilirubin concentration was considerably higher than that of the animals infused with the unbound pigment (Table III).

In the second series of experiments, all 25 guinea pigs were infused with bilirubin dissolved in guinea pig serum at a rate of 150 μg per g body weight per hour, which could be expected to result in considerable pigment deposition in the central nervous system (Figure 2). Immediately after conclusion of the pigment administration, 12 animals were infused with saline; of these, nine died within 24 hours (Table III). The remaining 13 guinea pigs were treated with human albumin; this resulted in the survival of ten of these animals despite their significantly higher serum bilirubin concentration (Table III).

In both series of experiments, the brain of guinea pigs with severe or fatal neurologic disease contained focal deposits of yellow pigment in the basal ganglia and nuclear areas surrounding the fourth ventricle. By contrast, this was not observed in animals that remained well.

Discussion

The blood-brain barrier appears to possess some of the general properties of biologic lipoid membranes (44, 45). Thus, it is relatively permeable to nonionized lipid soluble molecules, but passage of polar organic substances of large molecular size is restricted (45). Moreover, exchange of nonpolar lipid soluble compounds between plasma and central nervous system may occur within minutes and appears to be determined largely by the degree of lipid solubility (45). The results of the present study are in agreement with these concepts. In response to alterations of the binding properties of the plasma, unbound lipid soluble bilirubin was shown to pass the blood-brain barrier rapidly and in either direction (Figures 2 to 4, 7). By contrast, bilirubin either firmly bound to human albumin or as the water soluble conjugate did not gain access to the central nervous system.

Unlike previous investigations that depended largely on qualitative identification of tissue-bound pigment by visual means (46), in the present study bilirubin was quantitated by radioisotopic techniques (30). Whereas this permitted accurate determination of the pigment concentration in the nervous system, limitations inherent in the experimental models used should be noted. To achieve the desired pigment concentrations in the plasma, excessive amounts of bilirubin were used. This required infusion of relatively large volumes of fluid, containing either unphysiologic amounts of bile acids or heterologous albumin. The most significant limitation was the inability to estimate directly the unbound plasma bilirubin fraction available for transfer into the brain. Unbound pigment infused into the circulation or pigment displaced from its binding sites on albumin very rapidly escapes into the tissues (26), including the brain (Figures 2, 4, 7). It was reasonable to assume, therefore, that at the end of the experimental period, virtually all bilirubin remaining in the circulation was bound to albumin. Whereas this made possible direct comparison of the pigment concentration in the brain with the level of bound bilirubin in the plasma, the magnitude of the unbound fraction could only be inferred.

Within these experimental limitations, it was shown that A) when all infused bilirubin was bound to albumin, accumulation of pigment in the central nervous system was very small (Figure 2) and neurotoxicity was absent (Table III); B) infusion of bilirubin in excess of the circulating binding capacity resulted in a severalfold increase of pigment concentration in the brain (Figures 2, 7), frequently associated with severe neurologic damage (Table III); C) similar findings were obtained when salicylate competitively displaced pigment from its binding sites on plasma albumin (Figures 4, 7); and D) bilirubin deposited in the nervous system could in part be returned to the circulation by increasing the binding capacity of the plasma (Figure 3), thereby substantially reducing the neurotoxicity of the pigment (Table III).

The effect of decreased pH on the pigment level in the central nervous system was investigated because of clinical (47–49) and experimental (50–52) findings which suggested that anoxia or respiratory acidosis may enhance the occurrence or severity of bilirubin encephalopathy. The data presented in Figures 5 and 6 support these observations in that acidosis was found to augment pigment accumulation in the brain. It is unlikely that this was due merely to increased circulatory rates, because although 5% CO_2 markedly enhances cerebral blood flow (53), animals exposed to this gas mixture exhibited neither respiratory acidosis (54) nor increased bilirubin levels in the brain. A possible explanation may be derived from analogous observations with other weak organic acids which, like bilirubin (55, 56), exhibit dissociation constants close to the range of physiologic pH. With these compounds, acidosis increases the nonionized lipophilic fraction available for passage into the nervous system; simultaneously, it reduces serum levels (18, 54, 57). A similar mechanism may explain the present findings with bilirubin, but in the absence of detailed information on the binding interaction of pigment with albumin at variable pH, this interpretation must remain tentative.

The findings in adult Gunn rats were qualitatively similar to those in newborn guinea pigs (Figure 7). This suggests that no fundamental differences exist in the function of the blood-brain barrier for bilirubin between newborn and adult animals. Similar conclusions have been reached from studies with other compounds (58–63), and an operational blood-brain barrier has been dem-

onstrated *in utero* (59, 60, 63). These findings challenge the concept of "immaturity" of the blood-brain barrier that has been postulated (64) to explain the almost exclusive occurrence of bilirubin encephalopathy in icteric newborns (2–4). Plausible alternative explanations for this clinical observation are the extreme rarity with which severe unconjugated hyperbilirubinemia is encountered in later life (11, 65), the frequent occurrence of profound acidosis during the neonatal period (66), and the tendency to reduced plasma albumin concentrations in newborn, and particularly in premature, infants (66). Competitive displacement of bilirubin from its albumin binding sites may play an additional role, but it is difficult to assess because it involves not only exogenous compounds but also endogenous metabolites, including fatty acids (6, 27) and possibly other organic anions (67, 68). These factors, individually or in combination, probably account for the observation that the development of bilirubin encephalopathy cannot be predicted precisely from the serum bilirubin level (46). The theoretical possibility that albumin in newborns is different and may bind bilirubin with less affinity than the adult protein has been explored *in vitro* with negative results (69).

Although the exact process by which bilirubin damages the nervous system is not understood, the results of the survival studies (Table III) combined with the observation that virtually all radioactivity in the brain was present in the form of bilirubin-^{14}C leave little doubt that bilirubin rather than related compounds (9, 70, 71) is responsible for the neurotoxicity. In the newborn guinea pigs analysis of the pigment level in different anatomical regions of the brain failed to reveal significant differences. This contrasted with the findings in the adult Gunn rats, in which the highest bilirubin concentrations were found in the cerebellum and the brain stem. The main difference between these two experimental models was that the adult animals had pre-existing and irreversible neurologic damage (5, 72), and it appears plausible that damaged brain tissue has a greater propensity to bind or retain bile pigment that has crossed the blood-brain barrier (46, 50, 52, 73). It is also possible that selective areas of the brain are a priori more vulnerable to bilirubin and, once damaged, retain the pigment more avidly. This may account for the observation that in the newborn guinea pigs,

after the initial diffuse uptake of bilirubin by the brain, localized pigment deposition (kernicterus) was found in animals that developed "clinical" evidence of bilirubin encephalopathy. In a different experimental setting, a similar sequence of relatively diffuse uptake progressing subsequently to regional brain damage has been reported with a lipid soluble gas (74, 75). Such a sequential pattern is consistent with the previous observation (5) that shortly after birth, the brain of Gunn rats shows diffuse yellow staining, whereas after a few days of life, pigmentation is more prominent in certain nuclear masses. It also may explain why in infants with severe neonatal hyperbilirubinemia, the brain substance occasionally exhibits a diffuse icteric tint, rather than the usual regional yellow pigmentation (7, 46).

The present findings in animals provide firm experimental support for some of the prophylactic and therapeutic approaches that have been advocated for the prevention of bilirubin encephalopathy in human infants. The proposal to treat severely icteric newborns with albumin alone or in conjunction with exchange transfusion (76, 77) would seem to rest on a sound physiologic basis, in spite of the disquieting observation that the procedure tends to increase temporarily the serum bilirubin level (26, 76). The deleterious effects of respiratory or metabolic acidosis recently have been re-emphasized (49, 78) and the present study confirms the need for its early correction. Finally, as illustrated by the findings with salicylate, the importance of withholding sulfa drugs and recognizing other substances that may displace bilirubin from albumin has been confirmed experimentally (6, 22, 26, 27, 79).

Summary

Experiments were conducted in animals to study the effect of altered bilirubin-binding properties of the plasma on the pigment level in the brain. The experimental design was based on the findings that in the plasma virtually all bilirubin is bound to albumin, human albumin has a higher affinity for bilirubin than does albumin of rats or guinea pigs, and certain organic anions compete with the pigment for common binding sites on albumin.

In newborn guinea pigs and adult Gunn rats, bilirubin-^{14}C was infused either bound to human albumin or dissolved in aqueous taurocholate or

guinea pig serum. We found that *a*) administration of unbound bilirubin-^{14}C resulted in lower serum concentrations and higher brain levels of radioactivity than when the pigment was given bound to human albumin; *b*) bilirubin-^{14}C deposited in the brain by infusion of unbound pigment could in part be mobilized and returned to the circulation by subsequent treatment with human albumin; *c*) salicylate competitively displaced bilirubin from plasma albumin binding sites and enhanced accumulation of the pigment in the brain; and *d*) induction of acidosis increased the extravascular distribution of bilirubin-^{14}C, including the brain.

In newborn guinea pigs increased bilirubin concentration in the brain frequently was associated with severe neurologic damage and reduced viability. In animals infused with unbound pigment, the survival rate was substantially increased by subsequent treatment with human albumin, despite higher serum bilirubin levels produced by the albumin infusions.

These findings indicate that only unbound bilirubin can cross the blood-brain barrier. Consequently, the amount of bilirubin transferred into the brain is determined by the magnitude of the unbound pigment fraction rather than by the total pigment concentration of the plasma. The study provides direct experimental support for the proposed use of albumin in the prevention and treatment of bilirubin encephalopathy in neonatal hyperbilirubinemia, alone or in association with exchange transfusion. It also demonstrates the importance of early correction of acidosis and the deleterious effect of compounds that may displace bilirubin from albumin.

Addendum

Since the manuscript was submitted, the authors have learned that a patient with the Crigler-Najjar syndrome, who initially escaped detectable neurologic damage (80) despite severe unconjugated hyperbilirubinemia, developed striking neurological signs at 15 years of age. At death 6 months later, the brain showed changes characteristic of kernicterus (81). This unusual observation probably represents the first documented occurrence of bilirubin encephalopathy beyond the neonatal period, and it is in keeping with the results of this experimental study.

Acknowledgment

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