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PEDIATRIC RESEARCH

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The Effects of Brain Blood Flow on Brain Bilirubin Deposition in Newborn Piglets

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ABSTRACT. Since kernicteric lesions are usually found in the subcortical regions of the brain and these areas also receive the highest blood flow during asphyxia and hypercapnia, we hypothesized that increases in brain bilirubin deposition may be related to increases in brain blood flow. Fourteen piglets underwent a 3-h infusion of bilirubin to maintain total serum bilirubin at approximately 8 mg/dl, during which time blood gases, hemodynamic variables, and brain blood flow were determined. After sacrificing the animals, regional brain bilirubin content was determined. Ten piglets underwent the same protocol; in addition, hypercapnia was induced during the last hour of study ($PaCO_2$ approximately 70 mm Hg). The regional brain blood flow and bilirubin deposition were significantly increased over control values ($p < 0.05$) following hypercapnia in the subcortical region and significantly so in the midbrain and cerebellum. In separate groups of control ($n = 6$) and hypercapnia ($n = 6$) piglets, ^{125}I-labeled albumin was infused and demonstrated that hypercapnia was not associated with increased regional brain albumin content. We conclude that hypercapnia-induced augmentation in regional brain blood flow is associated with increased deposition of unbound bilirubin. Although the causal relationship between these two observations has not been firmly established, the findings deserve future investigation to clarify the role of brain blood flow, brain bilirubin deposition, and the production of kernicterus in high risk infants. (*Pediatr Res* 19: 691–696, 1985)

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Kernicterus has been described in low birth weight infants with relatively low levels of serum bilirubin concentration (1–5). Although the reason for this increased risk has been unknown, several hypotheses have been proposed. Blood-brain barrier integrity may differ between full term and low birth weight infants, although some investigators have questioned this concept (6). The reduced serum bilirubin-binding capacity observed in preterm infants may partly account for their increased risk of kernicterus as compared with full-term infants (7). Postmortem examinations of kernicteric infants have revealed regional differences in bilirubin staining. The subcortical areas such as thalamic nuclei, globus pallidus, caudate nucleus, and brainstem have demonstrated more extensive staining than the outer cortex (2, 8–10). Regional differences in brain blood flow have also been demonstrated in several laboratory animals including rats (11), newborn lambs (12), piglets (13), and cats (14). These differences were observed under baseline resting conditions. Furthermore, when challenged with hypercapnia, accentuation of these regional differences were observed both in piglets (13) and in cats (14) with higher blood flow to the subcortical regions than to the cerebrum. In recent studies, we have also demonstrated a flow dependent nature of brain bilirubin deposition (15).

Therefore, on the basis of increased frequency of subcortical staining with bilirubin in kernicteric lesions, the finding that these same regions have marked increases in blood flow during hypercapnia as compared with the cortical regions, and the flow-dependent nature of brain bilirubin deposition, we hypothesize that brain bilirubin deposition is augmented by increases in brain blood flow. The purpose of this study was to examine the regional changes in brain blood flow induced by hypercapnia and its relationship to regional brain bilirubin content in newborn piglets.

MATERIALS AND METHODS

Twenty-four 2- to 4-day old farm-bred piglets were divided into two study groups: a control group ($n = 14$, weight = 1.40 ± 0.09 kg, mean ± SEM) in which normocapnia was maintained throughout the study and an experimental group ($n = 10$, weight = 1.27 ± 0.07 kg, mean ± SEM) in which hypercapnia was induced. Within the control group, we performed regional brain blood flow measurements in six piglets and did not in eight in order to assess the potential influence of microsphere injections on the regional brain bilirubin content. As can be seen in Table 1, we found no difference in regional brain bilirubin contents between the two groups. Thus, the two groups were combined as one control group for data analysis.

Each piglet was removed from its sow on the morning of the study. Surgical procedures were performed under nitrous oxide inhalation anesthesia and local 1% xylocaine anesthesia. The left ventricle was catheterized via the right brachial artery for radionuclide-labeled microsphere injections using a Sentinel line catheter (inside diameter 0.67 mm, outside diameter 1.05 mm, Argyle, St. Louis, MO); the placement was verified with pressure tracings and by autopsy. Polyvinyl catheters (inside diameter 0.58 mm, outside diameter 0.99 mm, Bolab, Lake Havasu City, AZ) were also inserted into the following: 1) left brachial artery for microsphere reference blood withdrawal, 2) abdominal aorta via the right femoral artery for monitoring heart rate and mean arterial blood pressure and, 3) the inferior vena cava via the right femoral vein for bilirubin and in some animals for ^{125}I-labeled albumin infusion. The left common carotid artery was surgically exposed and two strands of 2–0 silk surgical suture were loosely placed behind the artery to allow for rapid access of the artery for catheterization at the termination of the study for *in situ* brain perfusion. Each piglet was removed from the nitrous oxide and placed in a specially designed darkened box for the study, which permitted access to the catheters without disturbing the piglet.

Following a 1-hr period for stabilization and recovery from surgery, baseline determinations were made on the awake, unrestrained piglets. Each was then given a 1-min bolus infusion of 12 mg/kg body weight of unconjugated bovine bilirubin (Sigma Chemical Corp., St. Louis, MO) dissolved in 5.4 ml of a buffered solution.[1] This was immediately followed by a continuous infusion of 10 mg bilirubin/kg/hr (167 mg/100 ml solution) for 3 hr. Preliminary studies indicated that this bilirubin dose maintained the piglet serum bilirubin at approximately 8 mg/100 ml for the duration of the study.

Table 1. *Regional brain bilirubin contents in the two control subgroups (mean ± SEM)*

	Bilirubin content (μg/g)	
	Without microspheres ($n = 8$)	With microspheres ($n = 6$)
Cerebrum	1.6 ± 0.3	1.8 ± 0.3
Thalamus and caudate nucleus	1.8 ± 0.4	1.9 ± 0.4
Midbrain	1.8 ± 0.2	2.5 ± 0.4
Cerebellum	2.1 ± 0.4	2.1 ± 0.4
Brainstem	2.7 ± 0.4	2.6 ± 0.6

During the study, each control piglet was awake and remained in an enclosed box breathing room air which was provided through portholes in the box walls. In the experimental group, the piglets were made hypercapnic ($Paco_2$ approximately 70 mm Hg) during the last hour of the study by the introduction of a gas mixture of 15% CO_2, 21% O_2, and the balance nitrogen through the portholes. Regional brain blood flow was determined at the baseline period, 120 (before hypercapnia), 150, and 180 min of the study period. Regional brain blood flow was measured using the technique described by Heymann *et al.* (17) with 15 ± 5 μm diameter microspheres labeled with one of the following radionuclides: ^{103}Ru, ^{57}Co, ^{113}Sn, ^{46}Sc, ^{51}Cr, or ^{95}Nb (New England Nuclear, Boston, MA). Approximately 6×10^5 microspheres suspended and continuously agitated in 2 ml of 10% dextran and 0.01% Tween were injected within 30 s into the left ventricle and flushed with 2 ml of normal saline. A reference sample of blood was collected continuously for 2 min beginning 15 s prior to the microsphere injection, using a constant withdrawal pump (Harvard Apparatus no. 940, Millis, MA) at a rate of 1.03 ml/min. Blood losses due to study sampling were replaced with blood from a young donor piglet. Arterial blood gases (Corning Blood Gas Analyzer 175, Corning Scientific, Medford, MA) were monitored at the baseline period, 60, 120, 140, 160, and 180 min following the onset of the bilirubin infusion. Mean arterial blood pressure and heart rate were continuously monitored using a pressure transducer (Hewlett-Packard 1280c, Waltham, MA) and recorded on a polygraph (Hewlett-Packard 7754A, Waltham, MA). Hematocrit levels were determined using a microhematocrit method.

At the termination of each study each piglet was anesthetized with nitrous oxide and local 1% xylocaine in preparation for left carotid artery catheterization with polyvinyl tubing (inside diameter 0.86 mm, outside diameter 1.32 mm, Bolab). The animal was then sacrificed with intravenous sodium thiamylal followed by a solution of saturated potassium chloride. The brain was then perfused *in situ* via the carotid artery with ice-cold normal saline for 15 min at a pressure of 60 mm Hg in order to remove blood from the brain vasculature and to reduce the breakdown of bilirubin by tissue peroxidases. In order to facilitate the perfusion the superior vena cava was incised, and the descending

[1] The buffered solution (pH 7.5) was prepared fresh on the day of each study and consisted of (by volume) 18.5% 0.1 N NaOH, 44.5% 5%-human serum albumin (Armour Pharmaceutical Co. Kankakec, IL), and 37% 0.055 M phosphate buffer. The bilirubin was first dissolved in the 0.1 N NaOH to which the albumin and phosphate buffer solutions were then added. The infusion apparatus containing the bilirubin solution was covered with aluminum foil to prevent light degradation of the bilirubin. This solution has previously been used in our laboratory for bilirubin studies in rats.

aorta was clamped to decrease the retrograde flow around the Circle of Willis and down the contralateral carotid artery. An autopsy was then performed to verify catheter placement and for removal of the brain. The brain was separated from the spinal cord at the level of the first cervical vertebra and divided into right and left hemispheres and weighed. The left hemisphere was divided into approximately 1-g sections representing the following brain regions: brainstem, cerebellum, midbrain, thalamus + caudate nucleus, and cerebrum (four representative sections of cerebrum taken: frontal lobe, occipital lobe, temporal lobe, and parietal lobe). The sections from the left hemisphere were immediately weighed and placed on ice. These sections were assayed within 3 h of the termination of the study for tissue bilirubin using a modified diazo method following chloroform extraction (18). The right hemisphere was anatomically divided in exactly the same manner as the left and placed into counting vials to an approximate height of 1 cm. Radioactivity of these tissues and of the reference blood samples were measured in a gamma well counter (Packard Autogamma Scintillation Spectrometer, Packard Instruments, Downers Grove, IL). All tissues and blood samples contained a minimum of 400 microspheres. The regional brain blood flow data was calculated, using a computer (PdP-11/34 Digital Equipment Corp, Maynard, MA) to correct for spillovers, according to the formula:

$$\text{brain blood flow (ml/min)} = \frac{\text{cpm of microspheres in the brain tissue}}{\text{cpm of microspheres in the reference blood}} \times \text{rate of withdrawal}$$

All values for regional brain blood flow were expressed as ml·min^{-1}·100 g^{-1}. Total serum bilirubin was measured by Martinek's modification of the diazo method of Malloy and Evelyn (19). Serum unbound bilirubin was estimated by the peroxidase method (20). Colorimetric determinations were performed on a single beam spectrophotometer (Gilford model 240, Oberlin, OH) and where appropriate were recorded on an automatic recorder (Gilford model 6051, Oberlin, OH).

Regional brain albumin content was measured in separate groups of six control and six experimental piglets treated in exactly the same manner as previously described except regional brain blood flow measurements were not determined. Regional brain albumin content was measured by intravenous infusion of 50 μCi of ^{125}I-bovine albumin (New England Nuclear) just prior to the bilirubin bolus injection. At the termination of the study, serum was obtained for ^{125}I-radioactivity. The serum albumin concentration (g/100 ml) at 180 min was also determined using the bromcresol green spectrophotometric method (20). The specific activity of the serum albumin was calculated:

$$\text{specific activity (cpm/}\mu\text{g albumin)} = \frac{\text{serum radioactivity (cpm/ml)}}{\text{serum albumin concentration (}\mu\text{g albumin/ml)}}$$

^{125}I-radioactivity of the eight sections of right hemispheric brain tissue as previously described, was determined using the same gamma well counter and corrected to cpm/g of brain tissue. The albumin content was then calculated as follows:

Table 2. *Arterial blood gas and hemodynamic variables (mean ± SEM)*

			Bilirubin infusion			Hypercapnia (E group)		
		Baseline	60	120	140	150	160	180
pH	C*	7.47 ± 0.01	7.49 ± 0.01	7.50 ± 0.01†	7.50 ± 0.01†	ND‡	7.49 ± 0.01	7.49 ± 0.01† (13)
	E	7.42 ± 0.01§	7.44 ± 0.01§	7.46 ± 0.01†·§	7.17 ± 0.01†·§	ND	7.16 ± 0.01†·§	7.15 ± 0.01†·§
P_{ACO_2} (mm Hg)	C	35 ± 1	33 ± 1	32 ± 1†	33 ± 1	ND	32 ± 1†	33 ± 1 (13)
	E	40 ± 2§	38 ± 1§	36 ± 1§	70 ± 1†·§	ND	71 ± 1†·§	76 ± 1†·§
P_{AO_2} (mm Hg)	C	75 ± 3 (13)	75 ± 3 (13)	73 ± 4	74 ± 3	ND	72 ± 2	71 ± 3 (13)
	E	78 ± 2	80 ± 2	81 ± 2	117 ± 3†·§	ND	117 ± 3†·§	115 ± 3†·§
BE (mEq/liter)	C	3 ± 1	3 ± 1	3 ± 1	3 ± 1	ND	2 ± 1 (13)	3 ± 1 (13)
	E	2 ± 1	3 ± 1	3 ± 1	−4 ± 1†·§	ND	−4 ± 1†·§	−3 ± 1†·§
Heart rate (beats/min)	C	198 ± 10	196 ± 7 (12)	209 ± 7 (13)	207 ± 8 (13)	210 ± 7 (13)	215 ± 9† (13)	221 ± 8† (13)
	E	(13) 194 ± 6	208 ± 6 (9)	203 ± 6	221 ± 15† (4)	228 ± 8†·§	223 ± 6† (4)	217 ± 8†
MABP (mm Hg)	C	69 ± 3 (12)	66 ± 3 (12)	69 ± 3 (12)	68 ± 3 (12)	71 ± 3 (12)	72 ± 3 (12)	66 ± 3 (12)
	E	61 ± 2	67 ± 2 (9)	70 ± 2†	67 ± 4† (4)	66 ± 2	62 ± 3 (4)	64 ± 1
Hematocrit (%)	C	24 ± 1	23 ± 1	22 ± 1†	ND	ND	ND	22 ± 1† (12)
	E	25 ± 1	24 ± 1	24 ± 1	ND	ND	ND	23 ± 1†
Total serum bilirubin (mg/dl)	C	1.6 ± 0.3	6.9 ± 0.3†	7.9 ± 0.3†	ND	8.6 ± 0.5†	ND	9.4 ± 0.4†
	E	0.4 ± 0.1 (8)	7.1 ± 0.3† (8)	7.9 ± 0.5†	ND	7.5 ± 0.4†	ND	8.0 ± 0.4†
Serum unbound bilirubin (nmol/liter)	C	Not detectable	205 ± 28†	205 ± 31†	ND	377 ± 53† (13)	ND	307 ± 43† (13)
	E	Not detectable	262 ± 38†	303 ± 31† (9)	ND	337 ± 57†	ND	446 ± 69†
Serum albumin (g/dl)	C	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1†	ND	1.7 ± 0.1†	ND	1.7 ± 0.1†
	E	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1 (9)	ND	1.4 ± 0.1†	ND	1.5 ± 0.1†

* C, control group, $n = 14$ unless otherwise noted in parentheses. E, experimental group, $n = 10$ unless otherwise noted in parentheses.
† $p < 0.05$ compared to baseline.
‡ Not determined.
§ $p < 0.05$ compared to control.

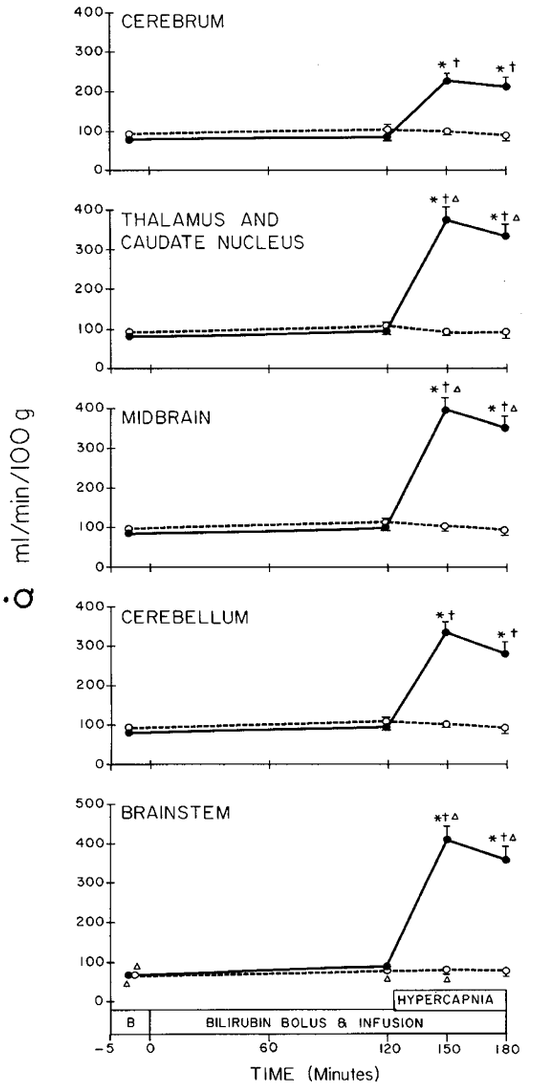

Fig. 1. Regional brain blood flow. Regional brain blood flow *versus* time (*B*, baseline) for the control group, *open circles* ($n = 6$) and the experimental group, *closed circles* ($n = 10$). All values are expressed as mean ± SEM. *$p < 0.05$ as compared to control, †$p < 0.05$ as compared to baseline with the same group. Δ $p < 0.05$ as compared to cerebrum value during the same time period.

$$\text{brain albumin content } (\mu\text{g albumin/g brain}) = \frac{\text{brain tissue } ^{125}\text{I-radioactivity (cpm/g brain)}}{\text{specific activity of albumin (cpm/}\mu\text{g Albumin)}}$$

Within group data were compared using the analysis of variance for repetitive measures. Where a significant difference was found, the Dunnett's multiple range *t* test was used to compare the means to the baseline values (22). Within group regional brain differences were analyzed using analysis of variance and the Newman-Keuls multiple comparison test. Between group analysis was done using the unpaired Student *t* test; where repetitive measures were compared between groups, the Bonferroni adjustment was used (23). Unless otherwise stated, a $p < 0.05$ was considered statistically significant. All values were expressed as mean ± SEM.

RESULTS

The arterial blood gas values, heart rate, mean arterial blood pressure, hematocrit, total serum bilirubin, serum unbound bilirubin, and serum albumin during the 3-h study in the control and experimental groups are summarized in Table 2. The pH and pCO_2 values remained stable during the study in the control group. Small differences in these values were observed in the experimental compared to the control group during normocapnia. In the experimental group, during hypercapnia, respiratory acidosis was produced. Heart rates were similar in both groups; increases from baseline values were observed toward the end of the study. Total serum bilirubin, serum unbound bilirubin, and serum albumin values increased similarly from baseline within both groups.

Figure 1 illustrates regional brain blood flow for each brain region examined in the control and experimental groups. Regional brain blood flow remained stable in the control group throughout the study period. In the experimental group, the regional brain blood flows were similar to those of the control group during the normocapnia period. During hypercapnia the blood flow to all brain regions increased significantly above baseline. Furthermore, within the experimental group at 180 min, the blood flow to thalamus + caudate nucleus, midbrain, and brainstem were significantly higher than the blood flow to the cerebrum.

Figure 2 illustrates the regional brain bilirubin content for each brain region examined in the control and experimental groups. Within the control group, the brainstem bilirubin content was significantly higher than the cerebrum. In the experimental group, the bilirubin content in the midbrain and cerebellum was significantly higher than in the control group. Within the exper-

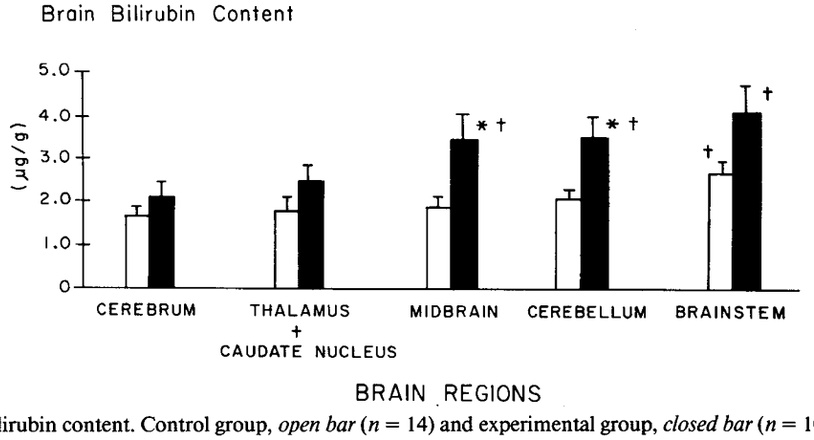

Fig. 2. Regional brain bilirubin content. Control group, *open bar* ($n = 14$) and experimental group, *closed bar* ($n = 10$). Mean ± SEM. *$p < 0.05$ as compared to control, †$p < 0.05$ as compared to the cerebrum value within the same group.

imental group, the bilirubin content in the midbrain, cerebellum, and brainstem was higher than in the cerebrum.

Figure 3 illustrates the regional brain albumin content in all regions examined in the additional groups of control and experimental piglets. The regional brain albumin content did not differ between groups in any region; however, for both the control and experimental groups the cerebellar albumin content was significantly higher than that of the cerebrum ($p < 0.05$).

DISCUSSION

We utilized the piglet for our study for a number of reasons: 1) cerebral blood flow has been studied in piglets extensively in our laboratory as well as others and regional brain blood flow has been demonstrated to be equal between hemispheres when it is expressed per unit weight of brain tissue (2, 24); 2) the piglet demonstrated significant increases in regional brain blood flow (13) with hypercapnia; and 3) the size of the piglet's brain provided easy dissection for the regional study of bilirubin content. Although regional brain bilirubin studies have not been done in piglets, several standardized measurements revealed that the modified technique of Bratlid and Winsnes (18) was feasible. The advantage of our surgical preparation was 2-fold: 1) there was minimal instrumentation to the brain itself and 2) the piglet was studied in its awake and intact state.

In preliminary studies, the effect of radionuclide-labeled microspheres on brain bilirubin deposition was examined. The lack of difference in regional brain bilirubin content between the two control subgroups indicated that 6×10^5 radionuclide-labeled microspheres given to these piglets did not alter the regional distribution of brain bilirubin. Furthermore, the results from the control piglets indicated that infusions of our bilirubin solution did not alter regional brain blood flow.

As noted in Table 2, there was a profound respiratory acidosis induced in the hypercapnic state in the experimental group. The elevation of the Pa_{O_2} in the experimental group during hypercapnia may be due to the Bohr effect with a decrease in pH noted during this time period. The heart rate increased during the latter part of the study in both groups; however, 30 min after the induction of hypercapnia the heart rate in the experimental group was significantly higher than the control piglets. The hypercapnia-induced transient increase in heart rate may result from the following possibilities: 1) hypercapnia has been shown to increase cardiac output (25) which is directly related to heart rate (26) and 2) hypercapnia has been shown to increase endogenous catecholamines (27). These results differ slightly from our previous work (13), perhaps because in that study the piglets were pancuronium-paralyzed. There was approximately an 8% decrease in hematocrit values in both groups despite blood replacement. The change in hematocrit values may have been a dilutional effect of the bilirubin infusion (6 ml/kg/h) along with repeated flushing of the catheters with small amounts of normal saline. Although cerebral blood flow is affected by hematocrit values, the hematocrit changes observed in our study were minimal and equivalent in both groups.

Serum total and unbound bilirubin values were maintained in the same range during the study period in both groups. The values obtained for the serum unbound bilirubin in our study group were approximately 10-fold higher than those obtained in human newborns (7), perhaps in part due to the low serum albumin concentrations found in the newborn piglets. The serum albumin levels in our study piglets increased slightly but significantly from baseline within both groups because of the infusion of human serum albumin throughout the study.

Regional brain blood flow measurements obtained in our piglets agreed with previous work in our laboratory. The experimental piglets had marked increases in brain blood flow to all regions during hypercapnia, and the increases were accentuated in the subcortical areas. Within the control group, the various regions of the brain had similar bilirubin contents, except for a slight but significant increase in bilirubin content in the brainstem as compared to cerebrum. The explanation for the latter observation was not apparent. The bilirubin contents of the various regions in the brain were generally higher during hypercapnia and significantly so in the midbrain and cerebellum (Fig. 2). This observation is associated with the increase in regional brain blood flow, but does not firmly establish the cause-effect relationship between blood flow and brain bilirubin deposition. These findings are of interest and deserve future investigation for consideration particularly in regard to the mechanism for such association.

Our study demonstrated no change in regional brain albumin content in the hypercapnia group. This is in contrast to that observed in a rat study in which a 2-fold increase in brain albumin content was found in the rat brains following induction of respiratory acidosis (16). The increased brain albumin content in that study may perhaps be secondary to: 1) the higher Pa_{CO_2} values achieved in the rat study cited above (120 *versus* 70 mm Hg in our study) and/or 2) possible species differences between rats and piglets. Based on our data, we conclude that the integrity of the blood-brain barrier to albumin in piglets was intact throughout the study and that the bilirubin deposited in the brain probably consisted mostly of unbound bilirubin. There is normally a minute amount of unbound bilirubin present in the circulation and in the brain which is reversibly in constant

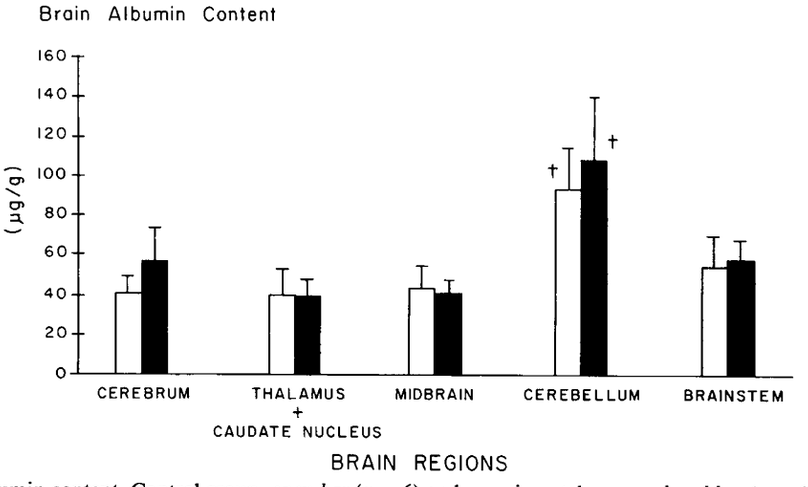

Fig. 3. Regional brain albumin content. Control group, *open bar* ($n = 6$) and experimental group, *closed bar* ($n = 6$). Mean ± SEM. †$p < 0.05$ as compared to the cerebrum value within the same group.

equilibrium between the plasma and brain cellular compartments. We have recently shown that the rate of transfer from the vascular to the tissue compartment (brain) is flow dependent (15).

In summary, hypercapnia in newborn piglets was associated with increased bilirubin deposition which was in part augmented by increased regional brain blood flow. The bilirubin deposited probably consisted primarily of unbound bilirubin, and the integrity of the blood-brain barrier was probably intact to albumin during hypercapnia.

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