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Autoradiographic Determination of Regional Cerebral Blood Flow in the Immature Rat

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ABSTRACT. Seven-day-old rats (15 g body weight) were injected subcutaneously with iodo-[^{14}C] antipyrine (5 μCi). After a variable period, each pup was decapitated and arterial blood collected for scintillation counting. Brains were immediately removed and either prepared for isotopic counting or frozen for autoradiography. The brain:blood partition coefficient was determined [0.944 ± 0.006 ml/g (mean ± SEM)]. Both cerebral blood flow (CBF) and regional CBF (RCBF) were calculated according to a formula derived from the Fick equation. CBF equaled 66 ± 4 ml/100 g/min (mean ± SEM), a value midway between reported 1-day-old rat CBF and adult rat CBF. Autoradiographs were of sufficient quality to permit microdensitimetric readings of a minimum of 11 structures. RCBF ranged from 20 ml/100 g/min in subcortical white matter to 71 ml/100 g/min in the brain stem. Immature rat RCBF, as a proportion of adult rat RCBF, was greatest in brain stem. Previously thought not feasible, this technique provides a reliable and relatively simple means of measuring both CBF and RCBF in the very small laboratory animal. (*Pediatr Res* 21: 471–476, 1987)

Abbreviations

CBF, cerebral blood flow
RCBF, regional cerebral blood flow
λ, brain:blood partition coefficient
IAP, iodo-[^{14}C] antipyrine
[^{131}I]CF_3I, ^{131}I-labeled trifluoroiodomethane

Interest in the neurologic problems of the human newborn (intraventricular hemorrhage, hypoxic-ischemic encephalopathy, cerebral infarction, kernicterus) has prompted numerous studies of CBF and RCBF (1–5). Although a variety of methods has been utilized to measure RCBF, all require a fairly large animal for study. The microsphere technique, originally described by Rudolph and Heymann (6), has been applied extensively to the study of lamb and piglet cerebral circulation (3, 7). This technique is limited to larger animals by the requirement for catheterization of major vessels. Autoradiographic techniques derived from work of Kety (8, 9) have also been used to measure RCBF in newborn animals, primarily immature dogs (10, 11). The need for vessel catheterization, as well as intraabdominal surgery for λ determination, makes successful application of this technique to small mammals unlikely.

We previously developed a model of hypoxic-ischemic encephalopathy in the immature rat. Following right common carotid artery ligation and exposure to an 8% oxygen environment, 1-wk-old rats develop reproducible brain damage (12). To determine the role of ischemia in production of this damage, we adapted one of the autoradiographic techniques noted above for RCBF determination in 7-day old rats. In the present report we describe application of this modified technique to the study of normal newborn rats. This successful application made possible measurement of RCBF in our model of hypoxic-ischemic encephalopathy (13).

MATERIALS AND METHODS

Dated pregnant Sprague-Dawley rats were purchased from a commercial breeder and allowed to deliver spontaneously. Newborn rats were kept with their dams up to a few minutes prior to experimentation, which took place at 7 days of postnatal age. Rat pups used in any given experiment were of similar weight, and where possible, from the same litter.

Each rat was injected subcutaneously with 5 μCi of IAP (53 mCi/mmol; New England Nuclear, Boston, MA). The injection was made into the back of the rat, roughly in the midline. Any observed bleeding or leakage from the injection site resulted in exclusion of the animal from the study (on average one to two animals per litter). After a variable time, the animal was decapitated and blood was collected from the trunk into a heparinized glass capillary tube. Ten μl of blood pipetted from the capillary tube was added to a scintillation vial containing 1 ml of Soluene-350 (United Technologies Packard, Downers Grove, IL). After mixing overnight in a mechanical shaker, the solution was combined with 9 ml of Dimilume-30 (United Technologies Packard). Samples then were counted on a Beckman LS-350 liquid scintillation counter.

The brains of some rat pups were removed from their skulls, and one or both cerebral hemispheres, sectioned at the pons-midbrain junction, were placed in scintillation vials with 1 ml of soluene-350 per 100 g brain weight. After mixing overnight, a 1-ml aliquot was added to 9 ml of Dimilume-30 and isotopically counted. Other brains were removed from their skulls and immediately frozen whole in liquid freon (−70° C). Coronal sections were cut at 20 μm thicknesses in a cryostat kept at −12° C. Sections were mounted on glass slides, dried, and subjected to quantitative carbon-14 autoradiography along with carbon-14 methylmethacrylate standards. These had been calibrated against standards for 20 μm dried brain tissue sections by the manufacturer (Amersham, Arlington Heights, IL). Comparison of optical densities of portions of the autoradiograms corresponding to specific brain regions with those of the carbon-14 standards yielded concentrations (mμCi/g) of IAP.

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Preliminary studies were performed to determine a postinjection interval during which blood and brain IAP concentrations were stable. Six 7-day-old rats then received 5 μCi of IAP subcutaneously and were sacrificed during this interval (20 min postinjection). Individual partition coefficients were calculated from brain and blood IAP measurements. The mean partition coefficient was used to calculate CBF as described below.

The primary goal of our initial experiment was to determine whether alteration of the injection-to-sacrifice interval altered calculated CBF. If calculated CBF did not increase or decrease with progressive changes in the study interval, then the interval could be chosen so as to optimize autoradiograph quality. In this initial CBF experiment, rat pups were sacrificed at predetermined intervals 15 s to 5 min postinjection. Blood was collected from each rat, while brains were removed only from those animals sacrificed at 1, 2, 3, 4, or 5 min. These brains were prepared for scintillation counting only. A total of 35 animals from three litters was used. Cerebral hemispheric blood flow was calculated for each interval (1, 2, 3, 4, or 5 min), using averaged values for blood and brain concentrations.

Subsequent experiments (n = 5) were performed on single litters to minimize the variability of animal size occurring among litters. Within each litter, individual rats were sacrificed at 15-s intervals postinjection, up to the 2-min endpoint. Additionally, three to five animals in each litter were sacrificed at 2 min. Brains, taken from this latter group only, were studied using either liquid scintillation counting or quantitative autoradiography.

Both CBF and RCBF were calculated according to an equation derived by Kety (8, 9):

$$C_B(T) = \lambda \cdot K \int_0^T C_A \cdot e^{-k(T-t)}\, dt$$

where: $C_B(T)$ = the concentration of tracer in brain at time T (dpm/g); λ = brain:blood partition coefficient (ml/g); K = flow/λ (min^{-1}); C_A = the concentration of tracer in blood (dpm/ml); T = time at end of experiment; and t = some time between 0 and T.

To solve the equation, a series of substitutions were made for K. The value of the integral was estimated using the trapezoid rule. Whichever K provided the calculated value of $C_B(T)$ closest to the observed value of $C_B(T)$ was the solution. This K, multiplied by λ, equaled flow.

Note: In Kety's original equation for inert gas exchange between blood and tissue, K was defined as follows: K = m · flow/λ where m was a constant between 0 and 1 that represented the diffusibility of a substance. In the absence of diffusion limitations, m was equal to 1 and therefore inconsequential in the relationship between K and blood flow to a given tissue. An inert gas, [^{131}I]CF_3I, was initially used for CBF determinations, in part because it had unrestricted diffusibility across the blood-brain barrier. Because CBF measurements performed with IAP are essentially equal to those made with [^{131}I]CF_3I, it has been concluded that no diffusion limitation for IAP exists in the brain (14).

RESULTS

Preliminary studies demonstrated that blood IAP concentrations plateaued at 5 min postinjection. Brain IAP concentrations did not plateau until approximately 10 min postinjection. Once peak concentrations were achieved, both blood and brain values remained unchanged up to 30 min. Brain and blood IAP concentrations measured 20 min postinjection in six animals were used to calculate individual brain:blood partition coefficients. The mean brain:blood partition coefficient was 0.944 ml/g with a SE of 0.006.

Individual measurements for our initial CBF experiment are shown in Figure 1. There was a rapid increase in blood concentration of IAP between 15 and 30 s postinjection. The rate of rise in blood concentration tapered off by 2.5 min. Brain concentration showed its most rapid rate of rise between 1 and 2 min and began to level off by 4 min.

CBF was calculated for each of the five time intervals in our initial experiment using averaged blood and brain concentrations. No systematic difference in flow calculations was found. Such a difference would not be expected given the low flow values measured (15). The 2-min interval was chosen as the study period for subsequent experiments in order to maximize contrast on the autoradiographs. The brain has no diffusion limitation to IAP; thus IAP entry is flow dependent. As study period length is increased, each area receives a greater cumulative flow. As more and more brain regions approach equilibrium for IAP concentration, diminished range of density on the autoradiograph should result. Use of the 2-min time interval allowed us to avoid this source of error.

Relevant "2-min" data from the initial CBF experiment described above were combined with data from all subsequent studies. One blood concentration curve was determined for each of the eight litters studied. Hemispheric brain concentration was measured in 14 rat pups. Comparison of each brain concentration to its litter-matched blood curve provided 14 CBF measurements. Individual CBF measurements are recorded in Table 1. Mean CBF equaled 66 ml/100 g/min with a SEM equal to 4.

For calculation of individual RCBF values, regional brain concentrations from 14 rat brains were matched with their litter-appropriate blood concentration curves (total five). Mean RCBF data for the 11 regions studied are reported in Table 2. The greatest measurable flow was to the brainstem while flows to cortex and the basal ganglia were intermediate. As expected, white matter flow was the lowest of all analyzed structures.

Examples of rat brain autoradiographs are shown in Figure 2. Only large readily identifiable areas were used for RCBF calculations. Many small structures (inferior colliculus, oculomotor nucleus, superior olivary nucleus) could be identified but their size prohibited accurate microdensitometric readings. The hippocampus showed a particularly dense pattern of isotope accumulation. Although not quantifiable, flow to this structure was clearly greater than flow to any of the structures listed in Table 2.

DISCUSSION

Seven-day-old rats weigh approximately 15 g. As a result, catheterization of major vessels is not feasible. Therefore a number of modifications in the indicator diffusion technique were required. First, isotope was administered subcutaneously as a bolus rather than as an intravenous infusion. IAP, administered subcutaneously, takes longer to appear in arterial blood than isotope given intravenously. The subcutaneous route is also associated with a more gradual increase in blood concentration. Fortunately the exact form of the arterial curve is not critical. The curve does increase monotonically; therefore, the requirements of the method are met.

Second, blood was not collected from a catheterized artery, but rather from severed neck vessels. As a result, it is not possible to rule out some contamination of carotid and vertebral artery blood with venous blood. In order to determine the degree of contamination, oxygen saturation of blood collected from the severed trunks of six 7-day-old rats was measured. Oxygen saturation averaged 85%, indicating the blood was primarily of arterial origin. We then attempted to estimate the effect of potential contamination on calculated blood flow. CBF calculations were made using reduced values for a hypothetical blood concentration curve. A range of brain concentrations were employed. If all values in the blood concentration curve were reduced 10%, then CBF values were increased only 7% (range 6 to 9%). Given the near normal oxygen saturations observed, we concluded that any error produced by this blood collection method must be small.

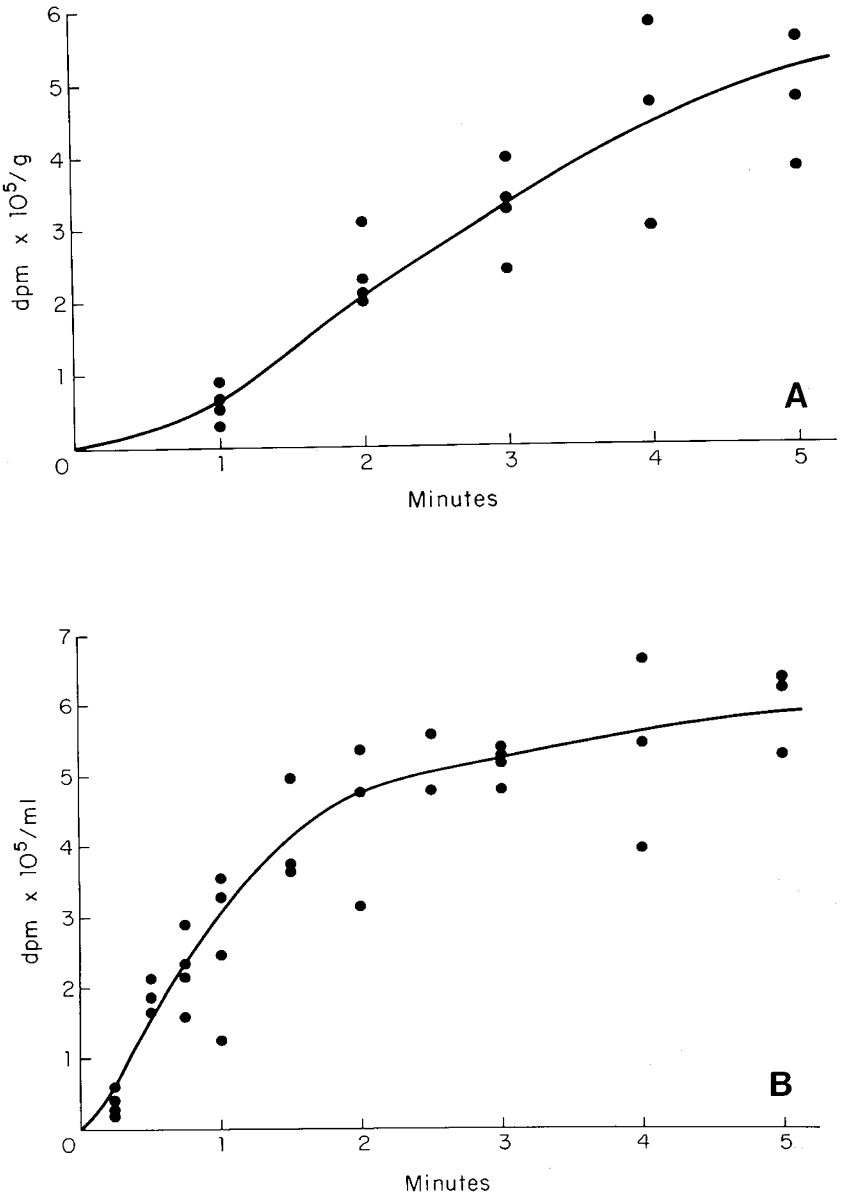

Fig. 1. Brain (*A*) and blood (*B*) concentrations of IAP over a 5-min period beginning immediately postisotope injection. Study animals were 7-day-old rats.

Equally important, the error should be consistent. It is likely that the same degree of venous contamination occurs with each blood collection. Therefore interanimal comparisons should be valid. All flow calculations, whether hemispheric or regional, should be equally exaggerated. This assumption of equal venous contamination becomes irrelevant when we consider intraanimal comparisons. All regional data in a given animal will be derived from the same blood curve.

Third, blood concentration curves were determined from groups of rats rather than from a single rat over time. Only intermediate-sized littermates were studied in a given experiment. Average weight was approximately 15 g with a range from 13 to 17 g. Because some animals were larger or smaller than the average, blood concentrations in those animals would have to be lower or higher than under ideal circumstances. To estimate the effect of this random variation, CBF was calculated for a typical blood concentration curve. Then the blood curve was modified so that alternate blood concentration values were either increased or decreased by the same proportion. This modification produced no change in calculated CBF.

It is worth noting that the adaptations we have employed in order to quantify newborn rat RCBF are not unique to our study. Fuglsang *et al.* (16), faced with the same inability to cannulate arteries and veins, similarly modified the integral method. In this study an arterial blood concentration curve was reconstructed from blood collected at decapitation of a series of newborn rats (16).

Our derivation of λ requires some additional comment. Typically circulation to the liver and kidneys is interrupted in order to prevent metabolism or excretion of the tracer. We did not ligate the portal vein, the hepatic artery, the mesenteric arteries, and the renal arteries because these procedures are technically very difficult and would have certainly disrupted rat pup homeostasis, possibly invalidating the partition coefficient measurement. Instead, we measured the partition coefficient while brain and blood concentrations were stable (and presumably in equilibrium). Excretion of the isotope is irrelevant as long as blood levels have been stable for a prolonged period prior to partition coefficient determination. Metabolism of the isotope is potentially a problem (conversion to radioactive metabolites with different chemical properties), but newborn rats have little oxidizing enzyme activity for antipyrine (17). Furthermore, the time

in which metabolism could have occurred was very short.

Our determination of λ in the 1-wk-old rat, 0.944 ml/g, corresponds to previously reported values. Adult rat λ equals 0.78 ml/g (14) while 3- to 12-h-old rat λ is reported to be 1.00

Table 1. *Hemispheric CBF determinations in individual 7-day-old rats based on scintillation counting of brain IAP concentration 2 min postinjection*

	CBF (ml/100 g/min)		
Animal	Left hemisphere	Right hemisphere	Average
1	66		66
2	67		67
3	85		85
4	48		48
5	68	63	66
6	66	73	70
7	37	38	38
8	60	62	61
9	88	86	87
10	64	82	73
11	79	76	78
12	66	66	66
13	55	55	55
14	62	72	67
Mean			66 ± 4 (SEM)

Table 2. *Autoradiographic measurements of RCBF in 7-day-old and adult rats*

Present study (n = 14)		Sakurada (n = 6)	
Region	RCBF*	Region	RCBF*
Brainstem		Brainstem	
Medulla-pons†	71 ± 8	Pontine gray	102 ± 7
Cortex		Cortex	
Frontal	66 ± 5	Frontal	139 ± 9
Parietal	53 ± 5	Parietal	169 ± 13
Occipital	46 ± 4	Occipital	150 ± 12
Deep gray		Deep gray	
Ventral posterolateral nucleus	63 ± 7	Thalamus:ventral	139 ± 6
Thalamus	44 ± 3	Thalamus:lateral	160 ± 9
Hypothalamus	52 ± 5	Hypothalamus	86 ± 4
Caudate	42 ± 4	Caudate-putamen	137 ± 4
Putamen-globus pallidus	43 ± 5	Globus pallidus	75 ± 5
Cerebellum		Cerebellum	
Cerebellar hemisphere†	39 ± 5	Cortex	100 ± 8
White matter		White matter	
Subcortical	20 ±2	Corpus callosum	40 ± 4
		Internal capsule	49 ± 2

* Expressed in ml/100 g/min (mean ± SEM).
† This area studied in 11 animals only.

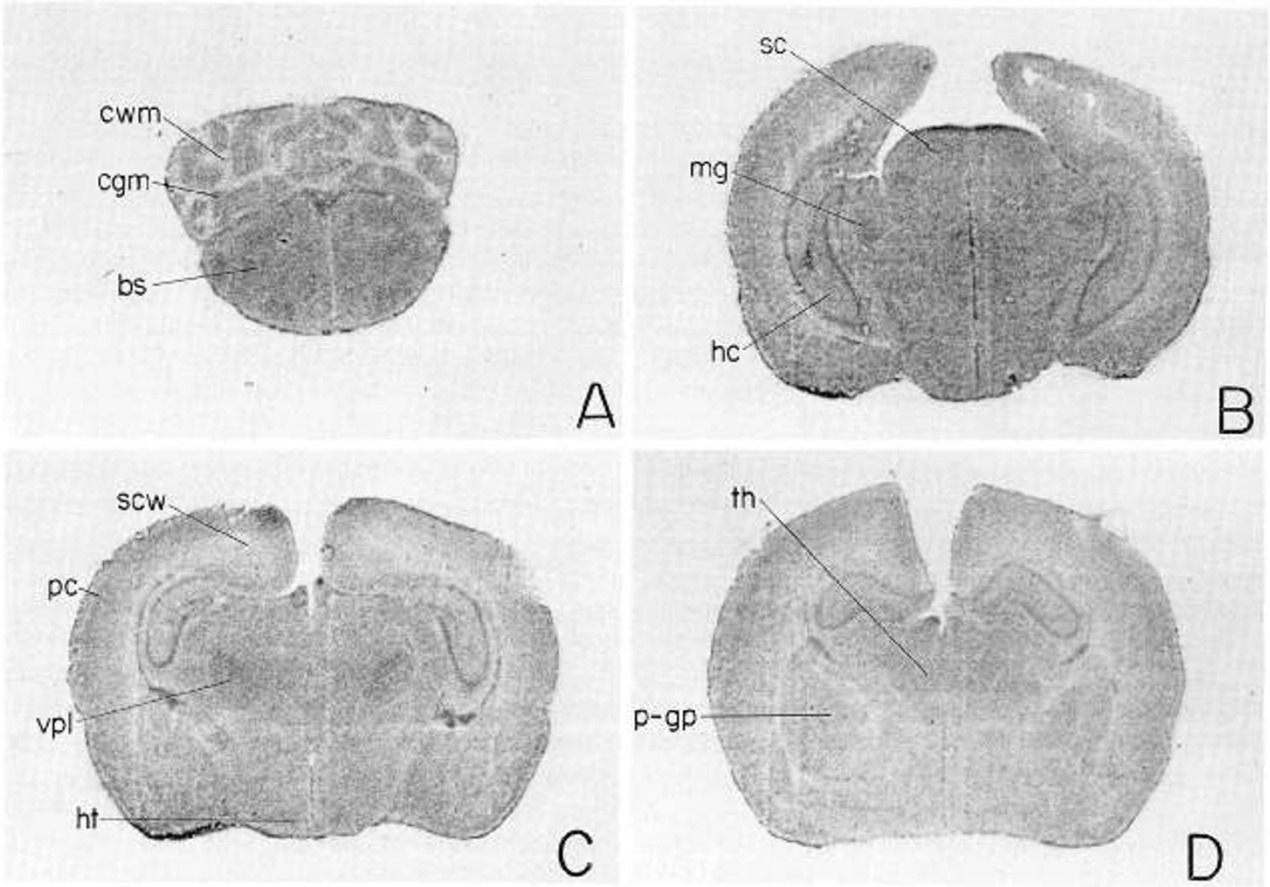

Fig. 2. Autoradiograms of coronal brain sections illustrating RCBF in a 7-day-old rat. *Darker regions* correspond to areas of greater flow. Sections shown include *A*, brainstem; *B*, midbrain; *C*, thalamus at the level of the VPL nucleus; *D*, a more rostral section of thalamus and basal ganglia. (*bs*, brainstem; *cgm*, cerebellar gray matter; *cwm*, cerebellar white matter; *ht*, hypothalamus; *hc*, hippocampus; *mg*, medial geniculate; *pc*, parietal cortex; *p-gp*, putamen-globus pallidus; sc, superior colliculus; *scw*, subcortical white matter; *th*, thalamus; *vpl*, ventral posterolateral nucleus).

ml/g (16). It appears that λ decreases with age in the rat. This is surprising since there is progressive lipid accumulation in the brain with advancing age and IAP is very lipid soluble. This decrease in λ with increasing age has not been seen in other animals. Notably, λ increases with age in the piglet (18).

Our hemispheric flow measurements bear comparison to earlier measurements of CBF in the rat (Table 3). Fuglsang *et al.* (16) using a technique similar to ours and employing IAP as the tracer, found CBF to equal 24 ml/100 g/min in the 3- to 12-h-old rat. Barker (19), measuring the rate of outflow of cerebral venous blood from the confluence sinorum, found CBF to equal 31.2 ml/100 g/min in the 1-day-old rat. Moore *et al.* (20) measured rat CBF using a method based on the permeation rate of tritiated water. They found CBF increased from 34 ml/100 g/min in the 1-day-old rat to 129 ml/100 g/min in the 14-day-old animal. Eklof *et al.* (15), using the Kety-Schmidt Xe^{133} inhalation technique, found adult rat CBF to equal 100 ml/100 g/min [see also Hernandez *et al.* (21)]. Clearly rat CBF increases over the first 2 wk of life and it is likely that this increase is a gradual one, corresponding to brain maturation. Our measurement of CBF at 7 days of age, 66 ml/100 g/min, supports this contention. Similar increases with age are seen in the dog, another animal with a relatively immature brain at birth (11, 22, 23).

The doubling in CBF found from birth to 7 days of age corresponds to a doubling in metabolic rate over the same time period. Duffy *et al.* (24) calculated rates of energy expenditure in rat forebrain under conditions of total ischemia. The energy use rate (Δ~P) increased from 1.33 mmol ~P/kg/min at 1 day to 2.58 mmoles ~P/kg/min at 7 days of age [where $\Delta\sim P = 2\cdot\Delta ATP + \Delta ADP + \Delta P\text{-creatine} + 2\cdot\Delta glucose + 1.45\ (\Delta\ lactate - 2\cdot\Delta glucose)$]. This suggests that CBF and cerebral metabolic rate are coupled in the developing rat.

While numerous investigators have studied RCBF in adult rats (25–28), no regional data have previously been reported for the immature rat. Sakurada *et al.* (14) studied RCBF in the adult rat using IAP as the tracer. Table 2 includes their results.

In analyzing our data we observed predominance of brainstem flow as expected for the immature brain (5, 10, 23, 29). This fact is highlighted when comparison is made to adult flow values. While most regions have flows that are approximately 40% of adult values, brainstem flow is already 70% of the adult flow. This difference in "blood flow maturity" may be even greater than the numbers indicate. Densitometric readings taken for brainstem and cerebellum undoubtedly include some areas of white matter. Adult values used for this comparison presumably were for gray matter only.

Marked labeling of the hippocampus was unexpected. Sakurada *et al.* (14) reported adult rat hippocampal flow of 100 ml/100 g/min. In the same study cortical flows ranged from 139 to 205 ml/100 g/min. It may be that relatively high hippocampal flow is seen only in immature animals. Review of neonatal dog RCBF data supports this notion. Batton *et al.* (30), Cavazutti and Duffy (10), and Shapiro *et al.* (31) all report hippocampal blood flow approximately equal to cortical blood flows.

In conclusion, we have demonstrated a technique for measurement of RCBF (and CBF) in very small laboratory animals. The technique requires few major resources, is simple to perform, and provides a reliable determination of blood flow to specific areas of immature rat brain.

Table 3. *Comparison of CBF data in rats*

Age	Technique	CBF (ml/100 g/min)	Reference
3–12 h	IAP	24	Fuglsang *et al.* (16)
1 day	Cerebral venous outflow	31.2	Barker (19)
1 day	Tritiated water	34	Moore *et al.* (20)
7 day	IAP	66	Present study
2 wk	Tritiated water	120	Moore *et al.* (20)
Adult	Kety-Schmidt Xe^{133}	100	Eklof *et al.* (15)
Adult	Kety-Schmidt Xe^{133}	103	Hernandez *et al.* (21)

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