Measurement of Local Cerebral Blood Flow in the Unanesthetized Rat Using a Hydrogen Clearance Method

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ABSTRACT

The hydrogen clearance method for measuring local tissue blood flow was adapted for use in the brain of the unanesthetized rat by means of a stainless steel plate screw mounted in the skull as the reference electrode instead of a calomel electrode. Blood flow was measured at 3 sites in the brains of 5 three-month-old rats over a 6-week period. Blood flow in the frontal cortex and the cerebellum for all the animals averaged 79 ± 22 (sd) and 81 ± 25 ml/min/100 g tissue, respectively. The other site, supposedly the hippocampus, exhibited a two-component clearance curve in most cases. The initial rapid component was of questionable validity; the second slow component averaged 110 ± 23 ml/min/100 g. Autopsy revealed that the hippocampal electrode was actually positioned in more than one type of tissue or proximal to an interspace. Hypoxia (PO₂ = 50 mm Hg) and hypercapnia (produced by inhalation of 10% CO₂) increased the clearance rates two- to four-fold. The rate of local blood flow and its variability were affected by the state of the animal; anesthesia (sodium pentobarbital) decreased the rate of blood flow as anticipated, and markedly reduced the variation in rate seen in the conscious state.

ADDITIONAL KEY WORDS cerebral cortex cerebellum hippocampus hypoxia hypercapnia anesthesia
facilitated by computer analysis and the authors stated that the quantitative aspects of the method required further study. Other workers (10) utilized a similar device to study changes in brain blood flow in unanesthetized rats, but made no attempt to correlate the electrical output of the thermocouples with actual blood flow in vivo.

In 1964, Aukland, Bower, and Berliner (11) adapted the hydrogen electrode to the measurement of local blood flow in myocardium and renal cortex of anesthetized dogs. The method is based upon the detection of hydrogen by a platinum electrode implanted at any site within the tissue of interest. Momentarily respired hydrogen is transported to the surface of the electrode where it produces a current proportional to the hydrogen concentration at the electrode. As the dissolved gas is cleared from the tissue and removed from the blood by the lungs, tissue hydrogen clearance is reflected by a decreasing current that is related to the rate of blood flow at the site of the electrode. Fieschi, Bozzao, and Agnoli (12) later used this same method to measure blood flow at various sites in the brain of the anesthetized cat. However, the well-known effects of anesthesia upon cerebral blood flow make any method requiring such treatment of limited value. In this report we describe a modification of the hydrogen-clearance method that permits chronic measurement of local blood flow in the brain of unanesthetized, unrestrained rats.

**Methods**

Measuring electrodes (Fig. 1) were fashioned from Teflon-coated 90% platinum–10% iridium wire, 0.007 inch in diameter (Medwire Corp., Mt. Vernon, N. Y.). One end of the wire was stripped of Teflon, soldered to a female micro-miniature pin connector (Amphenol Corp., Broadview, Ill.) and the wire was cut to the desired length. One millimeter of the tip of the electrode was bared of Teflon and plated with platinum black (11). The soldered joint and the points of removal of Teflon were coated with a plastic insulator (No-Arc Hi-Voltage Insulator, Chemtronics, Inc., Brooklyn, N. Y.). The reference electrode consisted of a miniature stainless steel plate screw (Bestfit, no. 5110/195 Jeambrun 23D, Sears, Roebuck & Co.) instead of the...
calomel electrode used by previous workers (11-13). Both measuring and reference electrodes were bathed in benzalkonium chloride (1:750) and rinsed with sterile distilled water just prior to implantation.

Male albino rats (Fischer 344, Charles River Breeding Laboratory) three months of age and weighing 240 to 290 g were anesthetized with sodium pentobarbital (4.0 mg/100 g body weight, ip). Chronic implants of three platinum electrodes were stereotactically set in each brain by use of dental cement (Kadon, L. D. Caulk Co., Milford, Del.). Coordinates according to the rat brain atlas of König and Klippel (14) were as follows: Site A (frontal cortex): A (frontal plane), 11.0 mm; L (sagittal plane), 1.5 mm; H (horizontal plane), 2.7 mm. Site B (hippocampus): A, 2.5 mm; L, 4.0 mm; H, 0 mm. Site C (cerebellum): P, 3.0 mm; L, 0 mm; H, 0 mm. Two plate screws were also mounted in the skull to serve as anchor posts for dental cement. One of these also served as the reference electrode by being attached to a pin connector with braided stainless steel surgical wire (Fig. 1). The first determination of blood flow was made approximately 24 hours after surgery.

The chamber in which the rats were placed for measuring blood flow was constructed from an aluminum desiccator, 8x8 inches (Fig. 2). It contained an upper compartment for the animal and a lower compartment where the gas mixtures were mixed with the atmosphere of the chamber, and the purging gas introduced. The oxygen tension in the upper compartment was measured continuously by means of an oxygen electrode (Beckman Model 777).

For each determination a single rat was placed in the chamber and the leads connected to the reference electrode and one of the measuring electrodes. The electronic system operating between the electrodes and a 10-inch recorder (1 mv full scale) is shown in Figure 3. The shunt resistor was usually set at 3000 to 4500 ohms and the 1000-ohm helipot was set so that there was no servo-deflection (balance point) when the circuit was closed prior to hydrogen administration. A typical measurement is shown in Figure 4. Initially the electrodes were balanced at some arbitrary baseline value on the recorder chart. The mixing fan was started and hydrogen was metered into the chamber through the manifold. At the same time, oxygen was metered into the chamber through the same manifold in quantities sufficient to maintain the desired oxygen tension in the upper compartment. When blood flow was measured under hypoxic conditions, the atmosphere was adjusted to the indicated Po2 with nitrogen 5 minutes before introduction of hydrogen. A 10% CO2-90% air mixture was used for the hypercapnia studies. In both situations, oxygen was added as required to maintain the Po2 at the desired level throughout the determination. Hydrogen flow to the chamber was stopped when the recorder response...
approached full scale, the glass cover on the chamber was raised slightly, and the purging gas turned on with sufficient velocity to quickly remove all hydrogen from the chamber. This purging was continued at reduced velocity throughout the rest of the recording. After duplicate recordings at each site had been made, the next electrode was connected to the circuit and the process repeated. In the cases of hypoxia and hypercapnia the atmosphere of the chamber was readjusted to normal for at least 10 minutes before the next site was studied. Local blood flow was calculated from each recording by use of the first-order rate equation, \( k = \frac{0.693}{t/2} \), where \( k \) has the dimension: ml/min/ml \((11)\). In order to do so, the hydrogen clearance curve was first replotted on semilogarithmic paper, subtracting the baseline value from each point on the recorded curve. The half-time, in seconds, for washout was determined from this graph and divided into the factor 4158 \((0.693 \times 60 \text{ sec} \times 100 \text{ g})\) to yield flow in terms of ml/min/100 g tissue. For the multi-exponential clearance curves from the hippocampal sites, the semilog plots were "resolved" into a "fast" and a "slow" component in the generally practiced manner of subtracting the secondary slope from the primary one.

Two to three months after implantation of the electrodes all five animals were anesthetized with sodium pentobarbital, the electrodes were removed and the total body perfused first with saline and then with 10% formalin. The solutions entered the left ventricle and left from an incision in the right auricle. The brain was removed and placed overnight in 10% formalin; frontal sections through each electrode site were then prepared by use of a Stadie-Riggs microtome blade.

**Results**

Inasmuch as the electrochemical properties of the platinum-stainless steel electrode system probably differ significantly from those of the platinum-calomel system \((15)\), it was necessary to ascertain whether the former produces quantitative data. Figure 5 shows the results obtained with an electrode in the brain when the rat was equilibrated with various concentrations of hydrogen in air. The broken line represents the theoretical proportional relationship between electrode response and hydrogen concentration; the open circles, the actual results. Despite the good agreement shown in Figure 5, we found in later experiments of a different nature that the response of the electrode was adversely affected by large changes in oxygen or carbon dioxide tension. To obviate this it is essential when purging the chamber of hydrogen to use a gas mixture having the same oxygen and carbon dioxide concentrations as the atmosphere in which it is desired to determine the rate of blood flow. If the concentrations are not the same, the recorder pen will not return to the same initial baseline due to electrode response to the change in oxygen concentration or pH.

The results obtained for the three sites studied in five rats during a six-week period are in Table 1. In the majority of cases the first of the duplicate determinations is larger than the second. Mean blood flows for sites A and C calculated from these data were 79 ± 22 (sd) and 81 ± 25 ml/min/100 g tissue, respectively, over the entire period. Most of the hydrogen desaturation curves

![Graph](image-url)
for the hippocampal site B were multi-exponential in nature. The half-times for the initial, fast component were very short (mean = 7.5 seconds) compared to those of sites A and C (52 seconds) and the slow component for site B (38 seconds). When calculated in terms of blood flow, 7.5 seconds corresponds to 544 ml/min/100 g tissue. This value is so far out of line with all known data on local tissue blood flow that it is obviously spurious. The slow component for this site had a mean of 110 ± 23 (SD) ml/min/100 g. In a second series of rats in which a shorter (4.0 mm) electrode was placed 2.0 mm anterior to, and 1.0 mm to the left of, site B, only mono-exponential clearance curves yielding a mean of 73 ± 15 (SD) ml/min/100 g (3 rats) were obtained. This region of the hippocampus corresponds most closely to the A3430 plane in König and Klippel (14).

Because of the observed discrepancy between duplicate determinations, several replication studies were carried out. Ten consecutive determinations of blood flow at site C in rat no. 2 made over a period of 80
minutes on day 2 yielded a mean of 77.9 ml/min/100 g tissue with a standard deviation of 2.3. A similar study of site A in rat no. 4 on day 42 yielded a mean of 79.5 ml/min/100 g tissue with a standard deviation of 2.2. The closed squares in Figure 6 illustrate the variability of local cerebral blood flow typically observed. The measurement made immediately after placing the animal in the chamber was almost always greater than the subsequent, more stable values unless the animal became unusually active or was intentionally disturbed. The mean of the first and second determination in this experiment was 84 ml/min/100 g tissue. The mean and standard deviation for the second through tenth measurements was 58.2 ± 6.2. When the rat was anesthetized following the initial measurements, the rate of blood flow underwent a further decrease as anticipated, and remained very stable (38.8 ± 2.1).

The effects of hypoxia and hypercapnia on brain blood flow at two of the sites in the five rats are summarized in Table 2. Although the two types of studies were carried out a week or more apart on any one rat, they were not performed at any particular time after the implantation except that days 1 and 2 were excluded. As expected, both hypoxia and hypercapnia resulted in an increase in the rate of hydrogen clearance.

Sketches of the unstained brain sections are shown in Figure 7. Sites A and C in rat no. 2 could not be defined because of mutilations occurring when the electrodes were removed. From Figure 7 it can be seen that sites A and C were quite uniformly placed with respect to the frontal and horizontal planes. Such was not the case for site B. Sites A and B are somewhat posterior to that intended due to the disparity between the size of the animals used in this study and the ones used by König and Klippel. As a result, the tip of the electrode at site A approached the forceps minor or the corpus callosum. For the same reason site B is closer to the midline than we desired. Instead of the electrode at site B being exclusively in the lateral aspect of the hippocampus, as intended, it was usually partially located in other structures. The cerebellar site (C), not shown by König and Klippel,
Sketches of brain sections showing location of 1-mm tip of electrode. The numbers refer to rat number, the letters to the site. Designations below sketches correspond to most similar frontal plane (in microns) in König and Klippel (14). Cerebellar sections are to a larger scale than forebrain sections.

was located in the vermian sublobule VIa (16).

Discussion

The only strictly quantitative study of local cerebral blood flow in an unanesthetized animal available for comparison with our results is that on the cat (3-5). Five areas of the cerebral cortex in ten cats exhibited mean local blood flows ranging from 77 to 138 ml/min/100 g tissue. This compares to an overall average of 79 ml/min/100 g (range, 36 to 156) for 71 determinations on the frontal cerebral cortex of the five rats in this study. Landau et al. (3) reported reductions in blood flow ranging from 24 to 53% in various cerebral cortical areas of cats anesthetized with thiopental. In the present study, cerebral cortical blood flow was reduced 33% by anesthesia if the mean of the quiescent values (58.2) is taken as the control. The cerebellar nuclei and cortex manifested average flow rates of 79 and 69 ml/min/100 g, respectively, in the cats (4) whereas the five rats showed an overall mean of 81 ml/min/100 g (range, 49 to 185) for 73 measurements. Average blood flow in the hippocampus of the cat was reported to be 61 ml/min/100 g (4). We observed a two-component hydrogen-clearance curve for the hippocampus in these five rats qualitatively similar to that reported by Fieschi et al. (12) for other parts of the cat brain, and as sometimes found with other inert gas clearance techniques (17, 18). Because of the uncertainty of the physiological interpretation of such multi-exponential curves (12, 19, 20), comparison to the results of the autoradiographic technique is of questionable value. However, it should be noted that the mean hippocampal blood flow in the rat as given by the slow component is within the range of values for other parts of the cat brain found by Landau et al. (3-5). Of course, the two methods do not necessarily measure blood flow in precisely the same sites within the brain. The values obtained by the autoradiographic technique are presumably the average for the entire cross-sectional area of the structure under examination whereas the hydrogen electrode supposedly measures flow only within the immediate
vicinity of the electrode. Since a structure such as the hippocampus or the cerebral cortex encompasses a rather large area in the brain it would not be surprising if there were variations in blood flow within each structure as, indeed, the autoradiographic technique shows. The mean hippocampal blood flow anterior to site B in the rat (73 ml/min/100 g) is in closer agreement with the values for the hippocampus of the cat (4).

The validity of the hydrogen-clearance method for measuring local blood flow in tissues has been examined and confirmed by Aukland et al. (11, 21) and by Neely and co-workers (13). However, in its original form the method is not usable for chronic studies with unanesthetized animals due to the requirement for a calomel reference electrode. This electrode, whether in direct contact with the animal or connected by means of a salt bridge, must either be applied to the skin or through an incision. A micro-miniature calomel electrode would still pose problems for chronic implantation. Rigid contact with tissue would be necessary to avoid frictional disturbance due to movement and the saturated KCl solution would probably have to be replenished frequently because of diffusion into the tissue. Such diffusion would in itself create problems if the electrode were mounted in the brain. Furthermore, we have observed considerable electronic "noise" between platinum electrodes in the brain and calomel electrodes attached to the skin. This noise is presumably due to the relatively large potential difference existing between the brain and blood (22) and makes an accurate analysis of the clearance curves impossible. Nevertheless, the advantages of a simple, effective reference electrode in the conscious animal are obvious. Although we have not examined the electrode characteristics of our platinum-stainless steel system, it does respond in a linear manner to the hydrogen concentration in the brain.

The sensitivity of the system to large changes in O₂ or CO₂ concentration is no great problem because the O₂ or CO₂ tensions must be kept constant to prevent a concomitant change in blood flow. A similar effect of O₂ was noted for the platinum-calomel system by Aukland et al. (11), who concluded that it would not seem to limit the usefulness of the method. We observed that a change in P_o₂ in the chamber from 155 to 50 mm Hg produced an electrode response of approximately 40 μV (4% of full scale), which remained constant so long as the new P_o₂ level was kept constant. Inasmuch as the new baseline was always established before administration of hydrogen, and the subsequent clearance curves returned to this same baseline as a result of keeping the P_o₂ constant, it is not likely that the electrode response to hydrogen during hypoxia is influenced by this condition. A similar situation prevailed during the hypercapnia experiments; that is, there was no change in the inspired P_co₂ or P_o₂ from the time the baseline was established until after the clearance curve returned to baseline.

Some instability or electrode "drift" was usually observed with fresh implants. This instability was sufficiently prolonged to prevent measurement of blood flow 24 hours after implantation in only one rat. In this connection it should be pointed out that plexiglass or other materials tending to accumulate static electricity cannot be used for construction of the chamber because the rush of purging gas through the chamber generates enough potential to interfere with the electrode current. The sensitivity of the electrodes gradually diminishes over a period of weeks or months. This may be due to the "aging" phenomenon observed for platinum black (23) or the corrosion that occurs on the plate screw. The length of time over which the electrodes remain useful also appears to be limited as much by the tendency of the animals to throw off the cranial attachment after several months. However, we have been able to follow some animals for as long as 4 months.

Ingvar and Lassen (17) have questioned the use of probes inserted directly into a
tissue for measuring blood flow, because the tissue is injured. This criticism was probably directed at the relatively larger probes required for detection of radioactivity. Aukland (21) studied the effect of tissue damage by needle-shaped electrodes upon tissue hydrogen clearance and concluded that it was not appreciable. From an investigation of proliferation of connective tissue around thermoelectric probes in the brain over a period of several weeks, Betz (24) stated that this factor was probably not of any importance. In the present study the lack of significant change in the flow rate over a six-week period at sites A and C supports these conclusions. Furthermore, in no case were any gross manifestations of tissue damage noted at autopsy. The decrease in electrode sensitivity with time in the brain is also observed in solution and thus is not specifically indicative of tissue reaction. The multi-exponential clearance curves, with the very rapid initial component noted in some cases, might suggest that they were artifacts resulting from tissue damage. However, Fieschi et al. (12) reported that tissue damage around the electrode tip reduces the washout rate of hydrogen. It is also noteworthy that these bi-phasic clearance curves were peculiar to site B and were not observed for the other sites that, presumably, are also susceptible to tissue damage.

The lack of reproducibility between duplicates on some occasions was the source of considerable concern until we realized that its occurrence coincided with an excited state in the rat. Neither physical exercise nor anxiety affects total brain blood flow in humans (25) but Betz (6) reported variations in cerebral blood flow measured by the heat-clearance method in conscious cats and dogs that did not occur if the animals were quiescent. Deliberate provocation of the animals increased the rate of heat clearance from the brain. Leatherman and Bean (10) also observed “prominent” fluctuations in cerebral blood flow (heat clearance) in some of their experiments, which did not occur when the animals were anesthetized. We have found that if a measurement is made immediately after a rat has been handled to connect the leads to the electrodes, the rate of blood flow is usually higher than during subsequent determinations, which tend to be reproducible. The effect is not due to the initial dose of hydrogen per se since a 5- to 10-minute waiting period between handling the rat and the initial measurement results in the lower, reproducible values. Pinching the rat’s paw with forceps accelerated hydrogen clearance but was followed by a return to the slower rate (Fig. 6). The fact that cerebral blood flow was constant during anesthesia, when the rat was insensitive to environmental stimuli, further attests to the validity of the measurements and the normalcy of variations in local cerebral blood flow in the conscious state. Thus it is plausible that any differences in local blood flow found in this study, compared to that of Kety’s group, may be because we did not attempt to control the behavioral factor whereas their cats were immobilized (3, 4) and therefore were probably quiescent. This being the case, the second of the duplicate values in this study should more accurately portray the “normal values” for local cerebral blood flow in unanesthetized rats. We now use separate leads to each platinum electrode, making it unnecessary to touch the animal when switching from one electrode to another.

The well-known response of cerebral blood flow to hypoxia and hypercapnia has been employed by numerous investigators to demonstrate the ability of the particular method under study to reflect changes in blood flow, and thus provide a test of the validity of the method. Freygang and Sokoloff (4) reported increases in cerebral cortical blood flow of the cat amounting to 1.2- to 2.1-fold, in response to 10% O₂ (corresponding to a Po₂ in inspired air of approximately 76 mm Hg) and about 1.5-fold with exposure to 5% CO₂. The corresponding increases for the cat cerebellum were about 2- and 1.5-fold for hypoxia and hypercapnia, respectively. In the present study the rats inhaled approximately 6.6% O₂.

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or 10% CO₂, and their hydrogen clearance increased about two- to four-fold in both the frontal cortex and cerebellum. Although the greatest increase in flow in these areas of the rat brain was approximately twice that found in the cat we have subsequently found that there is a difference of the same magnitude in the response of local cerebral blood flow in the rat to a Po₂ of 50 as opposed to a Po₂ of 75 in the inspired gas; the rate of blood flow in an atmosphere of 7% O₂ was nearly twice as great as that in 10% O₂. This is in close agreement with Betz’s findings in unanesthetized cats (26). A similar differential response to 10% CO₂ compared to 5% CO₂ was noted. Thus the difference between our findings with rats and the work of Freygang and Sokoloff with cats may simply be the difference in choice of gas concentrations. Whether the variation in responses we obtained for a given site is a function of the precise location of the electrode or only reflects differences in reaction among the rats is not known. Betz reported that inspired O₂ concentrations below 8% or CO₂ concentrations higher than 5% disturbed the normal behavior of cats and caused increases in cerebral blood flow unrelated to the effects of hypoxia and hypercapnia per se (26).

The occurrence of a two-component clearance curve at site B, although perplexing, is very interesting in view of the current controversy over the interpretation and significance of this phenomenon (19, 20, 27). Fieschi et al. (12) reported that their multi-exponential curves seemed to be characteristic of subcortical structures and the relative contributions of the slow and fast components was a function of the duration of hydrogen administration (degree of saturation) and clearance rate. Brief administration of hydrogen or experimentally induced increases in blood flow increased the relative weight of the fast component. Kjellmer and co-workers (18) stated that the shape of the 133Xe clearance curve from muscle varied systematically with the flow rate, tending to be mono-exponential at high flow rates. In the present investigation the bi-exponential curves were definitely associated with the proximity of the sensor to the demarkation between the outer and inner brain. Moreover, during hypoxia or hypercapnia all of the clearance curves at these sites were mono-exponential and of the same order of magnitude as the corresponding fast component during inhalation of air. A possible explanation for this phenomenon in the present case is that the electrode tip may be close to a cerebral blood vessel in this area of the brain. We have previously noted that the rate of hydrogen clearance from the abdominal aorta of the rat is extremely fast, the half-time being approximately 5 seconds (28). If the electrode tip is very close to an arteriole it would respond to both the very rapid arterial desaturation and the slower washout of the adjacent tissue. On the other hand, Landau et al. (3) and Kety (29) have cautioned against attempting to relate the rate of local blood flow directly to the degree of tissue vascularity.

The advantages of using this technique for measuring local cerebral blood flow in conscious animals are numerous. As pointed out by Aukland (21), the required electronic equipment is relatively simple. Calibration is unnecessary (11) and the calculations are not complex. Repeated determinations can be quickly made in the same animal under a variety of environmental conditions and pharmacological treatments. Only a single sub-surface probe is needed for measurement of flow at a given site by this method, whereas some of the heat-clearance methods require double probes placed contralaterally and equidistant to the midsaggital plane (6, 10). The diameter of the platinum electrodes in this study is smaller by a factor of two or more than that of the thermoelectric probes employed so far, thus further reducing possible trauma to the brain and the volume of the area recorded. By use of a multichannel circuit and recorder, blood flow at several sites can be measured simultaneously, limited only by the number of pairs of electrodes that can be accommodated by the dorsal aspect...
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of the skull. The necessity of supplying hydrogen to the brain may create difficulties in the application of the method to animals larger than cats and rabbits unless they are somehow restrained. Nevertheless, we believe that the technique has wide application and should greatly facilitate neurophysiological studies.

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References


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