Early Delineation of Ischemic Tissue in Rat Brain Cryosections by High-Contrast Staining

Johannes Vogel, MD; Christian Möbius, BSc; Wolfgang Kuschinsky, MD

Background and Purpose—After short periods of ischemia, commonly used staining methods yield only moderate differences in optical contrast between normal and damaged brain tissue when gray-scale images are used for computer-assisted image analysis. We describe a high-contrast silver infarct staining (SIS) method that allows an early delineation of ischemic tissue as soon as 2 hours after middle cerebral artery occlusion (MCAO) in rat brain cryosections.

Methods—Rats were subjected to permanent MCAO for 2, 4, 6, and 48 hours. The optical densities were quantified in nonischemic white and gray matter and in damaged tissue from gray-scale images of serial sections with the use of a video camera–based image analyzing system. SIS, hematoxylin-eosin, Nissl, and nitroblue tetrazolium stainings were performed in cryosections, and 2,3,5-triphenyltetrazolium hydrochloride (TTC) staining was performed in unfrozen vibratome sections. In addition, the range of reduced cerebral blood flow (CBF) in areas demarcated by SIS was determined in iodo[14C]antipyrine autoradiograms of adjacent cryosections.

Results—At all times after MCAO, only SIS showed significantly \((P<0.01)\) lower optical densities in damaged than in normal brain tissue for both white and gray matter. TTC staining was as effective as SIS 6 and 48 hours after MCAO. The tightest correlation between areas of reduced SIS and of reduced CBF was found at a mean ischemic CBF of 22.3 mL/100 g per minute. This corresponds to a CBF range of 0 to 44 mL/100 g per minute in areas of reduced SIS.

Conclusions—In contrast to other staining methods, SIS allows a reliable delineation of ischemic brain tissue (core plus penumbra) from nonischemic white and gray matter of rat brain cryosections as soon as 2 hours after MCAO. (Stroke. 1999;30:1134-1141.)

Key Words: cerebral ischemia, focal ■ histology ■ staining ■ tetrazolium salts ■ rats

Clinical studies have demonstrated the importance of an early therapeutic intervention after the onset of stroke. As a consequence, experimental models of stroke are used to demonstrate the effects of an early therapeutic intervention on the size of the brain lesion. To this end, the boundaries of the lesion are traced in black and white images of stained brain sections that have been acquired with the use of a video camera–based image analyzing system. However, gray-scale images of brain sections stained by conventional histological methods such as hematoxylin-eosin (H&E) or toluidine blue only show a moderate contrast between normal and damaged tissue, especially after short periods of ischemia. This contrast is lower than that which exists between gray and white matter in the normal nonischemic brain. Therefore, the lesion cannot be distinguished reliably from normal white matter structures by a lower optical density (OD) in black and white images of such stains. This results in an erroneous determination of the ischemic area with the use of computer-assisted image analyzing systems. The same problem, based on a lack of difference between the OD of normal white matter and ischemic brain tissue, is also typical of nitroblue tetrazolium (NBT) and 2,3,5-triphenyltetrazolium hydrochloride (TTC) stains. These stains are also commonly used for the delineation of ischemic brain tissue. NBT and TTC stains are based on the functioning of mitochondrial enzymes. Therefore, the intensity of these stains is related to the number of intact mitochondria. The low density of mitochondria in white matter structures results in a pale stain. This makes it impossible to discriminate between normal white matter structures and ischemic brain tissue in NBT as well as TTC stains. This is particularly true for the first hours after onset of ischemia. An additional disadvantage of TTC staining arises from the fact that only native, unfixed tissue can be used. Compared with native tissue, cryofixed tissue offers some advantages since the same cryofixed tissue sample can be used for simultaneous analysis by various techniques, such as histochemistry, histology, autoradiography, and molecular biology. Therefore, the present study aimed to develop an easy and rapid staining method suited for computer-assisted discrimination between normal...
and ischemic brain tissue by the use of gray-scale images obtained from stained cryosections. Such staining should result in large differences in OD between nonischemic and ischemic brain tissue and minimal differences between normal gray and normal white matter. To compare this staining with commonly used staining methods, the OD in gray and white matter and in the lesioned tissue should be determined in gray-scale images of H&E-, Nissl-, NBT-, and TTC-stained sections with the use of a computer-based image analyzing system. For the detection of the degree of oligemia and ischemia, local cerebral blood flow (CBF) should be measured within the damaged tissue as delineated by the new staining method with the use of quantitative iod[14C]antipyrine autoradiography.

Materials and Methods

Surgery
The experiments were performed in 24 adult male Sprague-Dawley rats in accordance with institutional guidelines. Twelve rats were used for histology and 12 for local CBF measurements. The animals were anesthetized by a gas mixture containing 1% to 1.5% halothane, 70% N2O, remainder O2. Body temperature was maintained at 37°C to 37.6°C with a temperature-controlled heating pad. Blood flow to the right middle cerebral artery was blocked by an intraluminal nylon thread (diameter 0.15 mm), which was covered with an elastomeric impression material (Provil, Bayer) at the end over a length of 10 mm, as described by Nagasawa and Kogure.19 Before the intraluminal suture was introduced, 12 rats were equipped with catheters inside the right femoral vein and artery to enable the intraluminal suture to be introduced, 12 rats were equipped with catheters inside the right femoral vein and artery to enable the infusion of iod[14C]antipyrine for the measurement of local CBF. After the incisions were closed, the anesthesia was withdrawn, and the animals were housed in cages with free access to food and water.

Histology (TTC, NBT, H&E, Nissl)
Twelve of the rats were killed 2, 4, 6, or 48 hours after middle cerebral artery occlusion (MCAO). The brains were cut immediately in a vibratome at +4°C into 500-μm coronal sections during superfusion with a Ca2+-free solution (mmol/L: NaCl 119.5, KCl 3, MgCl, 1, NaHCO3 24, NaH2PO4 1, glucose 10) that was bubbled with 5% CO2/95% O2. Alternating sections were placed either into 20-μm-thick brain sections (5 mL of a 10% silver nitrate solution. The formed precipitate was washed in distilled water 6 times for 1 minute before they were exposed to room air until it became copper colored (30 to 60 minutes). All solutions were prepared daily in carefully cleaned (65% nitric acid/distilled water) glassware.

 Autoradiography
Two of, 4, 6, or 48 hours after onset of MCAO, animals were placed into a rat restrainer (Braintree Scientific) for the measurement of local CBF by the autoradiographic method of Sakurada et al.24 as described elsewhere.25 In brief, 125 μCi/kg body wt iod[14C]antipyrine (Biocont) was infused with an increasing infusion rate for 1 minute. Parallel to this, 12 to 16 timed arterial blood samples were taken for the determination of the time course of the arterial iod[14C]antipyrine concentration. At the end of the infusion period, the animals were decapitated, and the brains were removed as quickly as possible and frozen in 2-methylbutane chilled to −60°C. Then the brains were embedded in M-1 embedding matrix (Lipshaw) and cut into 20-μm coronal sections at −20°C in a cryomicrotome. After they were dried on a heating plate at +4°C, the sections were exposed together with a [14C] standard set on a Kodak MinR1 x-ray film for 21 days. From the OD of the autoradiograms, local CBF was calculated with the use of an image analyzing system (MCID, Imaging Research Inc). In the autoradiograms, the size of the visible area of the total oligemic and ischemic tissue and the size of the areas in which the CBF was <30, 20, and 10 mL/100 g per minute were determined. Sections directly adjacent to those used for autoradiography were stained by SIS as described above.

Data Analysis
In the first experimental group, the different staining methods were compared. The OD in 3600 to 4000 pixels of gray and white matter as well as in the lesioned tissue was measured in adjacent SIS-, NBT-, H&E-, and Nissl-stained cryosections and TTC-stained vibratome sections of 3 rat brains 2, 4, 6, and 48 hours after MCAO, respectively, with the use of the aforementioned image analyzing system (charge-coupled device [CCD] camera used: Sony XL-77CE). The average OD measured in gray matter, white matter, and the lesioned tissue was compared with the multiple Student’s t test and Bonferroni correction. This analysis was performed for each different staining method. In the second experimental group, the autoradiograms were analyzed for areas of reduced blood flow. To this end, 4 areas of reduced blood flow were classified in each autoradiogram: the area of the total oligemic and ischemic tissue that was visible by a lowered OD and the areas that had CBF values ranging from 0 to 10, 0 to 20, and 0 to 30 mL/100 g per minute were determined. These areas were related to the area of infarcted tissue marked by SIS in adjacent cryosections with the use of linear regression analysis. The correlation coefficients obtained were tested for their difference from zero. The areas of damaged (SIS) and ischemic (autoradiography) tissue were determined by a blinded investigator. The level of statistical significance was set at P<0.05.
Results

The results of the OD measurements obtained by the different staining methods are summarized in Figures 1 and 2. Figure 1 gives examples of OD profiles determined by the different methods 2 or 48 hours after MCAO at the same level of consecutive serial sections. Exclusively in SIS sections, the contrast between gray and white matter is minimal in nonischemic tissue, whereas large differences exist in the OD between normal and ischemic brain tissue at all times after MCAO. In all other stains, the optical contrast between normal gray and normal white matter is higher or equals that which exists between gray matter and ischemic tissue, except for the TTC stain 6 and 48 hours after MCAO. Therefore, only SIS allows the delineation of ischemic brain tissue in gray as well as in white matter structures at all times after MCAO. The borderline between normal and ischemic tissue is clearly visible. OD values measured within the ventricle lumen are shaded because they are not relevant for the present findings. Width of the sections = 13 mm.

The Table shows the physiological variables of the rats subjected to local CBF measurement at different times after MCAO. All measured parameters remained unchanged. To assess the CBF in the areas of reduced SIS staining, the areas of reduced blood flow were related to the areas of reduced SIS intensity. Figure 3 shows typical iodo[14 C]antipyrine autoradiograms of cryosections together with the adjacent silver-stained cryosections taken 2 and 48 hours after MCAO. The areas of low perfusion in the autoradiograms correspond well with the areas of less intense staining in SIS. Therefore, the white line does not cross completely identical pixels in all sections. In contrast to all other staining methods, the OD in white and gray matter is the same in SIS-stained sections (a, f). In addition, the differences in OD between normal and damaged brain tissue are highest in SIS sections. The summary of these findings obtained from all different staining methods is given in Figure 2.

The relationship between the areas of lowered OD in the autoradiogram and SIS was quantified by linear regression analysis for different times and degrees of ischemia. Figure 4 shows 2 extreme examples of the correlations between the ischemic areas determined in numerous pairs of silver-stained sections and autoradiograms. One regression line refers to an early measurement 2 hours after MCAO. From the autoradiograms, areas have been selected in which CBF was <10 mL/100 g per minute. The correlation coefficient (r) of 0.62, although significant, indicates a considerable scatter of the data points around the regression line. For this correlation, the total oligemic and

Figure 1. Examples of OD profiles obtained from adjacent brain sections subjected to different staining methods 2 (a through e) and 48 (f through j) hours after MCAO. OD was measured along the white line crossing each section. The white line crosses comparable locations of gray and white matter in the nonischemic and the lesioned tissue in each section. The use of 20-μm (SIS, NBT) and 10-μm (H&E, Nissl) cryosections (SIS, NBT, H&E, Nissl) and unfrozen vibratome sections (TTC) resulted in slight irregularities of the different sections. Therefore, the white line does not cross completely identical pixels in all sections. In contrast to all other staining methods, the OD in white and gray matter is the same in SIS-stained sections (a, f). In addition, the differences in OD between normal and damaged brain tissue are highest in SIS sections. The borderline between normal and ischemic tissue is clearly visible. OD values measured within the ventricle lumen are shaded because they are not relevant for the present findings. Width of the sections = 13 mm.

Figure 2. ODs of nonischemic white matter (white bars) and the lesioned tissue (black bars) related to that of nonischemic gray matter (gray bars). For better comparison, the OD of gray matter was set to 100% for all staining methods at all times after MCAO. Exclusively in SIS sections, the lesioned tissue can be distinguished from both gray and white matter at all times after MCAO. Only SIS-stained sections showed the same OD in gray and white matter, whereas in all other stains white matter had a lower OD. NBT, H&E, and Nissl showed higher or equal ODs in the lesion than in nonischemic white matter up to 6 hours after MCAO; this was true for TTC only 2 hours after MCAO. With the exception of SIS-stained sections, significantly lower ODs between the lesion and white matter were only found in TTC-stained sections 6 and 48 hours after MCAO. *P < 0.05, **P < 0.01.

1136 Silver Staining of Infarcted Brain Tissue
Comparison of SIS (a, c) and [14C]iodoantipyrine autoradiography (b, d) 2 (a, b) and 48 (c, d) hours after MCAO. The areas of damaged tissue visible by reduced SIS (a, c) correspond well with the areas of low perfusion in the autoradiograms (b, d). In contrast to autoradiography, SIS (a, c) staining of nonischemic white matter is as intense as in nonischemic gray matter. This allows the exact delineation of ischemic tissue in white matter structures as well. For example, note the small tip of nonischemic white matter in the corpus callosum 48 hours after MCAO (c, arrowhead), which cannot be recognized in the corresponding autoradiogram (d). Bar = 5 mm.

Figure 3. Comparison of SIS (a, c) and [14C]iodoantipyrine autoradiography (b, d) 2 (a, b) and 48 (c, d) hours after MCAO. The areas of damaged tissue visible by reduced SIS (a, c) correspond well with the areas of low perfusion in the autoradiograms (b, d). In contrast to autoradiography, SIS (a, c) staining of nonischemic white matter is as intense as in nonischemic gray matter. This allows the exact delineation of ischemic tissue by SIS in white matter structures as well. For example, note the small tip of nonischemic white matter in the corpus callosum 48 hours after MCAO (c, arrowhead), which cannot be recognized in the corresponding autoradiogram (d). Bar = 5 mm.

Table 1: Physiological Variables at Different Times After MCAO in the Iodo[14C]antipyrine Experiments

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time After MCAO, h</th>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td>Arterial pH</td>
<td>7.406±0.04</td>
</tr>
<tr>
<td>Arterial Pco₂, mm Hg</td>
<td>42±5.9</td>
</tr>
<tr>
<td>Arterial Po₂, mm Hg</td>
<td>85±9.5</td>
</tr>
<tr>
<td>Base excess, mmol/L</td>
<td>1.3±0.8</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.46±0.05</td>
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</tbody>
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Values are mean±SD; n=3 in each group.

Figure 4. Two examples of the correlation between ischemic areas determined by SIS and autoradiography. Two hours after MCAO (a; y=0.98x+0.006, the data points are close to the line of identity (r and slope close to 1) when SIS-stained areas were correlated to the areas of the total oligemic and ischemic tissue (mean ischemic CBF of 22.3 mL/100 g per minute), indicating that both methods yielded highly corresponding areas of damaged brain tissue.
ischemic CBF of 22.3 mL/100 g per minute as detected from the reduced density in the autoradiograms corresponds to a range of blood flows from 0 to 44 mL/100 g per minute (Figure 6). Such an extrapolation was necessary since the direct measurement of areas displaying blood flow values between 0 and 44 mL/100 g per minute in the autoradiograms of ischemic tissue could be misleading: these areas could include normal, nonischemic white matter of corresponding blood flow values.25

Discussion

According to the present data, gray-scale images of SIS appear to be superior to other staining methods for tracing infarct size, especially at early times after MCAO. In contrast to all other staining methods tested, SIS showed a large and well-detectable difference in OD between damaged and normal brain tissue and no differences between white and gray matter as soon as 2 hours after MCAO. Comparison of the size of SIS-negative areas in brain cryosections with the corresponding area sizes of reduced CBF in iodo[14C]antipyrine autoradiograms 2 to 48 hours after MCAO showed a significant correlation of both area sizes for all times. The correlation became tighter at longer periods of MCAO and at higher blood flow values in the ischemic area.

In studies dealing with focal ischemia, lesion boundaries between normal and ischemic tissue have frequently been determined on the basis of the OD differences in black and white images of stained brain sections that have been acquired with a CCD camera connected to an image analyzing system.8,18 In such an analysis of sections stained by commonly used staining methods, the optical contrast between normal and ischemic tissue is low for several reasons: (1) Cellular changes such as edema and nuclear shrinking, when detected by conventional histological methods (eg, H&E or Nissl), can only be detected as moderate changes of the OD in gray-scale images. (2) Histological changes during ischemia often consist of a shift in the color distribution, which may not be detectable as a change in OD since different colors, eg, red and blue in H&E-stained sections, may result in similar gray values. This effect can be increased because the sensitivity of CCD cameras is proportional to the wavelength of the light at 600 to 700 nm. Therefore, the blue stains (eg, Nissl or NBT) in particular show a poor signal-to-noise ratio when acquired with a CCD camera. (3) The OD of nonischemic white matter is low (Figures 1 and 2). Since infarcted tissue is recognized by its reduced OD, it is almost impossible to distinguish between white matter structures and infarcted tissue with the use of image analyzing systems.8 As long as rat brain is used for studies of cerebral ischemia, the error of the determination of the ischemic area arising from white matter is small since rat

Figure 5. Correlation coefficients (a) and slopes (b) of the regression lines that describe the relationships between the areas of damaged tissue determined by SIS and by autoradiography (x axis) at different values of mean ischemic CBF (8.3, 11.2, 15.8, and 22.3 mL/100 g per minute; y axis) after different periods of MCAO (2, 4, 6, and 48 hours; z axis). a, Correlation coefficients of the regression lines. All correlation coefficients were significantly different from zero (P<0.01). The correlations between SIS and blood flow became tighter with higher ischemic blood flows as well as with longer periods of MCAO. b, Slopes of the regression lines. Slope values closest to 1 were found at a mean ischemic CBF of 22.3 mL/100 g per minute, indicating that the areas of damaged brain tissue determined by SIS are nearly congruent with the areas of low blood flow determined by autoradiography. At lower blood flows, the lower slopes indicate an overestimation of the ischemic areas by SIS.

Figure 6. Relationship between the ranges of reduced CBF and mean values of reduced CBF. This relationship was used to estimate the range of flow values that is detected by a reduced intensity of SIS. The basis of this estimation was the fact that at a mean CBF of 22.3 mL/100 g per minute, the areas of lowered staining by SIS were nearly congruent with the areas of low blood flow determined by autoradiography (Figure 3). A regression line was calculated for the mean ischemic CBF values measured in the areas in which CBF was <10, 20, or 30 mL/100 g per minute. The equation of this regression line (y=0.475x+1.6; r=1) can be used to calculate that a mean ischemic CBF of 22.3 mL/100 g/min corresponds to a range of blood flows from 0 to 44 mL/100 g per minute. The direct measurement of areas displaying blood flow values >35 to 40 mL/g per minute in ischemic tissue is misleading because it includes normal, nonischemic white matter (see Discussion).
brain contains ~10% white matter. For ischemic brains of other species such as cats or primates, which have a higher percentage of white matter, this appears to be a more serious problem.

In the present study the most commonly used staining methods were compared with SIS. To the best of our knowledge, no staining method exists that provides a significant optical contrast in gray-scale images between normal and ischemic brain tissue (gray and white matter) as soon as 2 hours after MCAO. In contrast to all other staining methods tested, the SIS method presented here yields the following advantages: (1) An equal stain of gray and white matter is found in nonischemic tissue and, at a lower OD, in ischemic tissue (Figures 1 and 2). Although the SIS method has not yet been tested in cats or primates, it appears likely that the same results can be obtained from these species. (2) A high optical contrast between normal and ischemic tissue is found in SIS sections 2 hours after MCAO, whereas such a high contrast is not obtained by NBT, H&E, and Nissl 48 hours after MCAO. (3) SIS is based on black and white staining, which makes it independent of the color sensitivity of CCD cameras. Thus, SIS allows one to distinguish ischemic from normal brain tissue earlier and more exactly than all other staining methods tested. Therefore, the lesion boundaries of the damaged brain tissue can be detected automatically with a high degree of accuracy that is not influenced by the subjectivity of the observer.8,18

Additional advantages of SIS derive from the fact that SIS can be performed in frozen tissue. Compared with TTC, which is frequently used for the detection of ischemic brain tissue, this has 2 consequences: (1) A higher spatial resolution can be achieved by SIS since thinner tissue slices (20-μm cryosections versus 0.5- to 2-mm vibratome/razor blade sections) can be obtained. (2) Since other methods of tissue processing are based on the use of cryosections or can be performed in cryofixed material, the SIS method enables the investigator to directly relate the infarct size to parameters of tissue function, such as histochemical, immunological, or perfusion-related data, which can be measured in adjacent cryosections. The detection of multiple parameters in adjacent brain sections within the same animal is especially useful in models of focal brain ischemia because the extent of infarction varies considerably from animal to animal.26

 Previously, thresholds of CBF have been defined to specify the pathological changes that occur in rat brain tissue after MCAO. Cerebral protein synthesis was found to be inhibited at CBF values <50 to 55 mL/100 g per minute 1 to 12 hours after MCAO.27,28 Tissue acidosis was detected at CBF values <30 mL/100 g per minute, whereas energy depletion started to occur at <15 to 20 mL/100 g per minute in rats subjected to 2 hours of MCAO.27-29 Therefore, it was of interest to define at which values of lowered CBF SIS intensity was reduced. Qualitatively, SIS was visibly reduced in all areas of clearly reduced CBF in the adjacent autoradiograms. Quantitatively, areas that had CBF values ranging from 0 to 10, 0 to 20, and 0 to 30 mL/100 g per minute were smaller than the corresponding lesion size determined in SIS sections, indicating that SIS includes CBF values >30 mL/100 g per minute. Inclusion of areas of CBF values ranging from 30 to 40 mL/100 g per minute would result in an erroneous inclusion of nonischemic white matter structures by the image analyzing program since nonischemic white matter has blood flows of ~35 to 40 mL/100 g per minute.25 Therefore, lesion size was determined in iodinated3Hantipyrine autoradiograms by manual tracing of the visible areas of the total oligemic and ischemic tissue (gray and white matter). For all times after MCAO, these areas (mean ischemic CBF, 22.3 mL/100 g per minute) closely correlated with the lesion areas determined in corresponding SIS cryosections. However, the question of which CBF range might correspond to a mean ischemic CBF of 22.3 mL/100 g per minute remained. Because of the potential inclusion of nonischemic white matter, the CBF range in the tissue displayed by SIS could not be determined directly. Therefore, the range of CBF in the area displayed by SIS was estimated with an extrapolation from the values of mean ischemic CBF of 6.3, 11.2, and 15.8 mL/100 g per minute, which were derived from the areas that had CBF values ranging from 0 to 10, 0 to 20, and 0 to 30 mL/100 g per minute. The mean ischemic CBF values were related to their corresponding CBF range (Figure 6). The equation of the resulting regression line was used to calculate the range of CBF values that corresponds to the mean CBF of 22.3 mL/100 g per minute measured in the total oligemic and ischemic tissue. This estimation yielded blood flow values ranging from 0 to 44 mL/100 g per minute within the area of reduced SIS intensity. Since the penumbra has been assigned to CBF values between 23 and 47 mL/100 g per minute,30,31 reduced SIS intensity appears to encompass the total ischemic and oligemic (penumbra) areas.

The present study shows that SIS is a sensitive staining method for the detection of oligemic and ischemic brain tissue at CBF values <44 mL/100 g per minute. In contrast to TTC, the new silver staining method can be applied to cryosections and is suited for the detection of oligemic and ischemic brain tissue as soon as 2 hours after onset of focal ischemia. This makes it possible to combine SIS with autoradiographic, histochemical, and other methods applied to adjacent cryosections of the same brain.

References
Silver Staining of Infarcted Brain Tissue

The authors describe a silver staining method, which, when coupled with the required image analyzing equipment, give an optical density signal that, by virtue of its pallor, distinguishes normal from ischemic rat brain. The method is used on frozen material, and in the present instance, employed frozen sections of the entire rat brain. The authors review other techniques for delineating ischemic from nonischemic brain. They report that the present technique can distinguish ischemic from nonischemic tissue at 2 hours, a period earlier than that reported for other techniques and earlier than that of the tetrazolium method. However, they have not used other species. The tetrazolium techniques are often used on gross (ie, not microscopic) slices of the brain. Using the entire brain in that way, the investigators are able to determine infarct volumes in large specimens. The silver method does not do this. Therefore, it would seem that its value is, in fact, limited to brains sufficiently small so that an entire hemisphere can fit on a slide after freezing and sectioning.

An important part of the study was the comparison of the staining results with radioautographic determination of blood flow. The authors discovered that pallor in the silver-stained sections corresponded to a wide range of reduced flows, including those used by others to define the penumbra. If this is correct, the authors have devised a method by which the entire “at-risk” zone of ischemia can be morphologically

1140


15. Nagasawa H, Kogure K. Correlation between cerebral blood flow and another type of glia, or they can stain neuronal cell bodies or their axons and dendrites, or they can stain blood vessels. The authors do not tell us what is being stained here, but it may be the axons, since the white matter and cortex are both optically dense until affected by ischemia, when both become pale and readily distinguished from surrounding normal tissue.

Second, the authors express the belief that the technique will be useful in animals with brains larger than those of rats. However, they have not used other species. The tetrazolium techniques are often used on gross (ie, not microscopic) slices of the brain. Using the entire brain in that way, the investigator is not dependent on microscopic sections and can delineate infarct volumes in large specimens. The silver technique does not do this. Therefore, it would seem that its value is, in fact, limited to brains sufficiently small so that an entire hemisphere can fit on a slide after freezing and sectioning.

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The new technique should be useful, especially for determining the size of the “lesion” in the period 2 to 10 hours after injury. However, the article raises interesting questions. First: what is being stained by the precipitated silver? Silver stains, depending upon the recipe used, can stain one or another type of glia, or they can stain neuronal cell bodies or

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delineated at a period as early as 2 hours after ischemia. This could then be compared with the volume of brain that actually dies with the passage of time, and investigators could determine whether, and how much of, the penumbra was saved. Two hours is an extremely early period at which to define the area at risk. Indeed, microscopic evidence of neuronal damage such as microvacuolation of neurons is difficult to detect even in carefully perfused fixed brain prior to 4 hours, and the much more readily recognized irreversibly injured eosinophilic or acidophilic neuron may not appear until 12 to 24 hours after the onset of ischemia, though some have reported seeing such neurons as early as 6 hours after ischemia. One wonders whether a careful comparison of the pale area in the present study, with conventionally stained sections, might reveal a heretofore unrecognized histological marker for cells that are already irreversibly committed to die or (less likely, I suppose) for cells that are still alive but malfunctioning in the penumbra. In this regard, it is important to point out that the new method, unlike some others, gives a strong change in optical density of white matter shortly after onset of ischemia. Because neuronal cell bodies are only present in gray matter, the signal in white matter must reflect staining of one of the other elements mentioned above. If that element is axons, and if the penumbra includes white matter (does it?), then the present study shows that axons of neurons in the penumbra are directly affected by ischemia and undergo some sort of reversible alteration marked by failure of silver staining or that some change in the neuronal cell bodies from which these axons arise has led to a change in the argyrophilia of the axons. For this reason alone it would be of great interest to pursue an understanding of the basis for the staining and its failure in this study.

William I. Rosenblum, MD, Guest Editor
Department of Neuropathology
Virginia Commonwealth University
Medical College of Virginia
Richmond, Virginia
Early Delineation of Ischemic Tissue in Rat Brain Cryosections by High-Contrast Staining
Johannes Vogel, Christian Möbius and Wolfgang Kuschinsky

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