Factors Limiting Regeneration of ATP following Temporary Ischemia in Cat Brain

Frank A. Welsh, Ph.D., Michael J. O’Connor, M.D., Val Robert Marcy, B.S., Anthony J. Spatacco, B.A., and Rebecca L. Johns, B.A.

SUMMARY Cerebral ischemia was induced in cats using bilateral carotid artery occlusion coupled with hemorrhagic hypotension. Thirty minutes of ischemia, which depleted levels of ATP and phosphocreatine throughout the cerebral cortex, was followed by 2-4 hours of recirculation. During the recovery period, cortical perfusion and NADH fluorescence were monitored through a cranial window. Postischemic perfusion, as indicated by transit time, was initially higher than control, but declined to subnormal levels by 60 minutes. NADH fluorescence transients, induced by brief anoxia, also decreased steadily during recirculation, indicating a failure of oxidation-reduction capability. The disappearance of anoxic-NADH transients usually preceded the decline of flow, suggesting that O₂ delivery was not the factor limiting redox reactions. Furthermore, tissue levels of NADH, which were nearly normal after 2-4 hours of recirculation, did not indicate tissue hypoxia.

In spite of normalization of NADH, resynthesis of high energy phosphates was severely impaired. The degree of ATP recovery varied widely in different cortical regions; however, there were two general groups of ATP values — one at 5% and the other at 70% of control levels. In the energy-depleted areas, NADH levels were normal, but the total pool of NAD (NADH + NAD⁺) and the tissue content of K⁺ were 43% lower than control. In contrast, the NAD pool and K⁺ content were only slightly diminished in the regions with greater ATP restitution. The results suggest that postischemic resynthesis of ATP may be limited not by inadequate delivery of O₂, but rather by defective production of NADH.

Permanently Disruption of energy metabolism occurs in several models of temporary cerebral ischemia. Two distinct mechanisms may account for this irreversible energy failure. First, postischemic blood flow may not be adequate for energy restitution even though the capability for ATP resynthesis remains. Alternatively, there may be a primary lesion within the cell which prevents resynthesis of ATP regardless of blood flow. Although postischemic hypoperfusion has been well demonstrated, it is not known whether flow plays a limiting role in ATP regeneration. If blood flow were delivering insufficient amounts of O₂, then the resulting tissue hypoxia should be accompanied by increased levels of NADH. However, tissue NADH levels were not higher than normal in regions with permanent energy depletion. In the present study we have measured the time-course of postischemic blood flow and of redox transients of surface NADH fluorescence. In addition, we have determined regional levels of NADH, NAD⁺, and K⁺ in recirculated brain and correlated these levels with postischemic restitution of ATP.

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Methods
Cats, weighing 2.5–4.0 kg, were anesthetized with ketamine (20 mg/kg), immobilized with gallamine, and ventilated mechanically with a mixture of 75% N₂O and 25% O₂. Respiratory rate and volume were adjusted to give an end-tidal CO₂ of 4.5%. Core temperature was maintained at 38°C using a heating lamp and rectal thermistor, and arterial pressure was recorded via a femoral catheter. The common carotid arteries were isolated and encircled with loose-fitting ligatures. The head of the animal was then placed into a stereotaxic frame, and a craniotomy (1.5 cm diameter) was made over the parietal cortex of the right hemisphere. The dura was removed, and a glass plate was fastened into the craniotomy at the level of the inner table of the skull. Electrodes were screwed into the skull in a quadrangular array for EEG recording.

Cerebral ischemia was initiated by occluding the carotid arteries and simultaneously withdrawing arterial blood into a heparinized syringe. Arterial pressure was lowered until the EEG became isoelectric, and this pressure (25–35 torr) was maintained by withdrawing or reinfusing small quantities of blood. After 30 minutes, ischemia was reversed by releasing the carotid snare and reinfusing the collected blood. During the recirculation period, arterial blood gases and pressure were measured, and systemic acidosis was corrected with intravenous bicarbonate in 22 of the 40 experiments.

Cortical perfusion was monitored through the cranial window by recording the cerebrovascular transit of Thioflavin S, a fluorescent, nondiffusible tracer.

A fluorometric method was chosen in order to compare perfusion directly with NADH fluorescence in the same volume of cortex. Since both NADH and Thioflavin S absorb light at 366 nm and fluoresce at 450 nm, the cortex was illuminated with ultraviolet light (366 nm) and the 450 nm fluorescence was recorded continuously with a television camera fitted with an image-intensifier. Cortical perfusion was measured during the control period and at intervals of 15–30 minutes during postischemic recirculation. For each measurement, 0.2 ml of 10 mg/ml thioflavin was injected into the arterial circulation either at the root of the aortic arch via a femoral catheter or into the left atrial appendage through a transthoracic catheter. The cortical transit time was estimated from the fluorescent transient by extrapolating to baseline the steepest portion of the ascending and descending phases of the transient, and measuring the intervening duration. In order to assess the reproducibility of thioflavin injection, a shunt between a femoral artery and vein was inserted in a few animals, and the transit of thioflavin through this shunt was recorded fluorometrically.

The capacity of the cerebral cortex to generate NADH was determined using transient hypoxia. Thus, upon completion of the flow measurement, the transient elevation of cortical NADH fluorescence was recorded during a brief interval (48 sec) of systemic anoxia. Systemic anoxia was produced by replacing O₂ with N₂ in the ventilation mixture; typically the anoxic increase of cerebral NADH occurred after a delay of 20–30 seconds. The intensity of 366 nm reflected light was also measured to detect changes in fluorescence quenching by hemoglobin. However, during postischemic recirculation, alterations of 366 nm reflectance during transient anoxia were negligible.

After 2–4 hours of recirculation, the brain was frozen in situ with liquid N₂ for regional measurements of metabolites. In 3 additional animals, the brain was frozen after 30 minutes of ischemia without recirculation. NADH fluorescence in frozen brain sections was recorded photographically as described previously. The regional distribution of ATP was determined in adjacent thin sections (30 microns) using a modification of the luciferin-luciferase technique.

Unfixed, frozen sections were layered onto the luciferin reagent at −15°C, melted at room temperature, and immediately placed in contact with Polaroid type 52 film for a 10-sec exposure. Although the use of unfixed tissue permits endogenous ATPases to compete with the luciferin reaction, the initial luminescent image was in general agreement with quantitative assay for ATP in regional samples (unpublished data and figures 3 and 4).

Regional brain pH was measured qualitatively using the fluorescent pH indicator, umbelliferone. Frozen thin sections were layered at −15°C onto strips of cellulose acetate which had been impregnated with umbelliferone. The layers were melted and illuminated with ultraviolet light (366 nm). The fluorescent image (450 nm), the intensity of which is directly proportional to pH, was recorded photographically on high contrast Polaroid type 51 film.

Regional samples, for quantitative measurement of tissue metabolites, were removed from sections of frozen brain used to measure NADH fluorescence with a small cork borer (2 mm diameter) in a −30°C glove box. After weighing (2–5 mg), the samples were extracted by one of two procedures. The first was designed for combined measurements of ATP, phosphocreatine, lactate, NAD⁺, and NADH. The sample was added to 50 μl of 0.1 N NaOH in methanol at −30°C, and a suspension was made with a glass stirring rod. In an ice-water bath, 1 ml of 0.01 N NaOH was added, and a 95 μl aliquot was made 1 mM in cysteine and heated for 10 min at 60°C to destroy NAD⁺. The heated aliquot was assayed for NADH. The remaining portion of the tissue suspension was acidified with perchloric acid, centrifuged, neutralized, and analyzed for ATP, phosphocreatine, lactate, and NAD⁺.

The second extraction method was designed for measurement of total tissue K⁺ along with ATP, phosphocreatine, and lactate. The sample was added to 50 μl of 0.1 N LiOH in methanol at −30°C, and a suspension was made before transfer to an ice-water bath. After adding 230 μl of 0.05 N LiOH, the mixture was heated at 95°C for 5 min to destroy tissue enzymes. LiOH was used rather than NaOH to reduce
the blank rate of pyruvate kinase activity in the subsequent assay for K+. This extraction method yielded values for ATP, phosphocreatine, and lactate from control brain identical to those obtained by acid extraction.

Enzymatic, fluorometric techniques were employed to assay ATP, phosphocreatine, and lactate, as outlined by Lowry and Passonneau.\textsuperscript{18} NAD\textsuperscript{+} and NADH were measured with an enzymatic cycling method.\textsuperscript{18} K\textsuperscript{+} was determined using the stimulation of pyruvate kinase as described by Outlaw and Lowry.\textsuperscript{17} Statistical comparisons were made using Dunnett’s modification of the i test.\textsuperscript{18}

Results

Arterial blood gases remained in the normal range during the recirculation period except for a slight increase of pCO\textsubscript{2} at 30 minutes (table 1). In 18 animals (out of 40) in which postischemic bicarbonate infusion was omitted, arterial pH fell to 7.06 ± 0.02 (mean ± SEM) at 30 minutes and recovered to 7.18 ± 0.02 at two hours. Despite this systemic acidosis, there was no difference of metabolite levels in brain between bicarbonate-treated and untreated animals. The arterial pressure declined significantly during recovery, but did not fall below a mean pressure of 100 torr, or the animal was discarded from the series.

Thirty minutes of ischemia depleted ATP and phosphocreatine throughout the cerebral cortex (table 2). Lactate was uniformly increased to extremely high levels in all cortical regions analyzed. However, the total content of K\textsuperscript{+} decreased only slightly during the 30-minute insult.

Following the period of ischemia, recirculation was measured at 15-minute intervals using the fluorescent tracer, Thioflavin S. Figure 1 illustrates the sequence of thioflavin transients recorded simultaneously from the cortical surface and from a femoral arteriovenous shunt. The fluorescence transients in the shunt did not vary during recovery, demonstrating the uniformity of the thioflavin arterial curve following each injection. At 15 minutes of recirculation, the amplitude of the cortical transient was larger than that of the preischemic trace, and the washout was more rapid. By 60 minutes, the cortical transient was similar to the control, but at 150 minutes, the amplitude was markedly diminished. Although the time-course varied in different animals, the gradual decrease of perfusion following an initial period of hyperemia was a consistent finding (table 3). Thus, at 15 minutes of recirculation, the transit time was slightly reduced, while the amplitude of the thioflavin transient was moderately increased compared to control values. However, by 60 minutes, transit time had increased by 50%, and the amplitude was no longer elevated. These trends of decreasing amplitude and increasing transit time continued at 120 and 180 minutes of recirculation.

However, preceding the decline of perfusion, there was a significant decrease in the amplitude of the anoxic-NADH transient, which is a measure of the capacity of the cortex to generate NADH. Thus, at 15 minutes, the NADH transient was only 59% of control and had diminished to 23% by 60 minutes. At all durations of recirculation, the percentage decrease of the NADH transient was greater than that for blood flow. Indeed, in individual animals, the anoxic-NADH transient disappeared at a time of relative hyperemia (fig. 2). After 45 minutes of recirculation in this animal, the amplitude of the NADH transient was less than 10% of control while cortical perfusion remained markedly increased.

Postischemic restoration of brain ATP levels varied greatly in different regions and in different animals. Topographic depiction of ATP revealed focal reductions, which correlated with regional alterations of pH and NADH tissue fluorescence (fig. 3). Regions with reduced ATP (A, C, and D) showed a remarkable depression of pH and NADH fluorescence. Quantitative measurements in these areas confirmed the decrease of ATP and NADH and demonstrated an extreme elevation of tissue lactate. However, the level of NADH was the same (11 \textmu mol/kg) in regions A and B despite differences in NADH fluorescence and ATP luminescence. There was a profound decrease of NAD\textsuperscript{+} in regions with impaired metabolic recovery.

Regions which failed to reconstitute ATP levels during postischemic recirculation were invariably acidic, but did not always exhibit diminished NADH fluorescence (fig. 4). In this brain, patches of intense NADH fluorescence were superimposed upon a darker background in areas depleted of ATP. Despite the increased fluorescence, the measured value of NADH (sample D) was lower than normal. Thus, the intense tissue fluorescence in this brain was not due to high levels of NADH. As in the previous example, tissue levels of NAD\textsuperscript{+} were markedly decreased in areas with energy failure.

Table 2. Cranial Altersations during Ischemia

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>PCr</th>
<th>Lac</th>
<th>K\textsuperscript{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.22</td>
<td>4.16</td>
<td>2.10</td>
<td>120</td>
</tr>
<tr>
<td>± 0.09</td>
<td>± 0.24</td>
<td>± 0.24</td>
<td>± 2</td>
<td>± 2</td>
</tr>
<tr>
<td>Ischemia:</td>
<td>0.05*</td>
<td>0.02*</td>
<td>36.9*</td>
<td>109</td>
</tr>
<tr>
<td>30 minutes</td>
<td>± 0.02</td>
<td>± 0.03</td>
<td>± 6.9</td>
<td>± 6</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

N = 3 animals per group, 2-6 samples per animal.

Values for ATP, PCr (phosphocreatine), Lac (lactate), and K\textsuperscript{+} expressed as mmol/kg.

*Different from control, p < 0.05.

Table 1. Arterial Variables

<table>
<thead>
<tr>
<th></th>
<th>Mean pressure (torr)</th>
<th>pO\textsubscript{2} (torr)</th>
<th>pCO\textsubscript{2} (torr)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control period</td>
<td>155 ± 3</td>
<td>110 ± 2</td>
<td>29.7 ± 0.5</td>
<td>7.35</td>
</tr>
<tr>
<td>Recirculation</td>
<td>153 ± 4</td>
<td>118 ± 4</td>
<td>33.1 ± 1.2</td>
<td>7.35</td>
</tr>
<tr>
<td>30 minutes</td>
<td>132* ± 6</td>
<td>122 ± 4</td>
<td>27.9 ± 0.9</td>
<td>7.43</td>
</tr>
<tr>
<td>2 hours</td>
<td>132* ± 6</td>
<td>122 ± 4</td>
<td>27.9 ± 0.9</td>
<td>7.43</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

N = 40 animals, except for pH where N = 22.

*Different from control period, p < 0.05.
ATP REGENERATION FOLLOWING ISCHEMIA/Welsh et al.

CEREBRAL CORTEX

REFERENCE ARTERY

CONTROL PERIOD

RECIRCULATION: 15 Minutes

RECIRCULATION: 60 Minutes

RECIRCULATION: 150 Minutes

THIOFLAVIN INJECTION

FLUORESCENCE INCREASE

15 Minutes

5 sec

THIOFLAVIN INJECTION

10.4 ± 1.1*

17 ± 11*

Figure 1. Thioflavin Fluorescence Transients from Cerebral Cortex and from a Reference Artery during Postischemic Recirculation. After bolus injection into the arterial circulation, the transit of Thioflavin S was recorded fluorometrically through a cranial window (CEREBRAL CORTEX) and simultaneously from a femoral arteriovenous shunt (REFERENCE ARTERY) prior to cerebral ischemia (CONTROL PERIOD) and at three different times during recirculation.

Although the regional restoration of ATP ranged widely in recirculated brain, a frequency histogram of the number of samples at a given ATP value revealed two major groups of samples (fig. 5). In one group, ATP levels were less than 0.5 mmol/kg, while in the other, ATP was resynthesized to levels between 1.3 and 2.0 mmol/kg. In the group of samples with low ATP, lactate remained at end-insult levels, but NADH was slightly lower than control (table 4). NADH levels were diminished by 43% resulting in an increase of the NADH/NAD⁺ ratio. The total tissue content of K⁺ was also 43% lower than control in regions with ATP less than 0.5 mmol/kg. By contrast, in areas which restored ATP above 1.3 mmol/kg, lactate levels were substantially reduced from end-insult levels, but remained elevated compared to control. Interestingly, NADH levels were 30% greater than control values, and there were minor decrements in

Table 3. Cortical Thioflavin Fluorescence Transients and Anoxic NADH Fluorescence Transients during Postischemic Recirculation

<table>
<thead>
<tr>
<th>Time</th>
<th>Thioflavin Amplitude (% of control)</th>
<th>Thioflavin Transit time (sec)</th>
<th>Amplitude of anoxic increase of NADH (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.1 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>105 ± 14</td>
<td>5.7 ± 0.4</td>
<td>59 ± 10*</td>
</tr>
<tr>
<td>30 min</td>
<td>90 ± 13</td>
<td>8.0 ± 1.0</td>
<td>38 ± 10*</td>
</tr>
<tr>
<td>60 min</td>
<td>72 ± 12</td>
<td>9.0 ± 1.0*</td>
<td>23 ± 9*</td>
</tr>
<tr>
<td>120 min</td>
<td>55 ± 9*</td>
<td>10.4 ± 1.1*</td>
<td>21 ± 8*</td>
</tr>
<tr>
<td>180 min</td>
<td>49 ± 8*</td>
<td>12.8 ± 1.4*</td>
<td>17 ± 11*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. N = 25 animals for thioflavin, except at 180 min. where N = 16. N = 9 animals for anoxic NADH. *Different from control, p < 0.05.
THIOFLAVIN FLUORESCENCE

NADH FLUORESCENCE

CONTROL PERIOD

Fluorescence Increase

20 sec

FIGURE 2. Thioflavin Fluorescence Transients and Anoxic NADH Fluorescence Transients in Cerebral Cortex during Postischemic Recirculation. Sequential fluorometric recordings through a cranial window of the transit of Thioflavin and of the anoxic elevation of NADH prior to cerebral ischemia (CONTROL PERIOD) and at three different times during recirculation.
Discussion

Models of temporary cerebral ischemia permit identification of irreversible alterations which may limit recovery from the ischemic insult. The central aim of the present report was to investigate the factors which interfere with postischemic restitution of brain energy metabolism. Since the initial demonstration of "no-reflow," postischemic blood flow has been implicated as a major factor preventing recovery of brain tissue. More recent reports have confirmed the occurrence of focal hypoperfusion following severe ischemia, but others have demonstrated significant neuropathology in the absence of impaired flow and have questioned the causative role of postischemic hypoperfusion in the production of ischemic damage. In the present study, similar to previous reports, initial postischemic hyperemia was followed by a steady decline of perfusion to subnormal levels. The critical question is whether this perfusion decrement causes a second and more permanent episode of tissue hypoxia. Evaluation of the adequacy of a given level of flow is difficult if the metabolic demand is not known. Thus, a subnormal flow may be sufficient if the metabolic rate is comparably reduced. Alternatively, if the delivery of \( O_2 \) were insufficient, then the tissue would become hypoxic and levels of NADH would increase. However, the present study demonstrates that NADH was not elevated in areas permanently depleted of ATP. Furthermore, the NADH/NAD\(^+\) ratio, while elevated, was the same as that in regions with higher ATP levels. Therefore, postischemic \( O_2 \) delivery does not influence the tissue content of NAD\(^+\) and K\(^+\). The NADH/NAD\(^+\) ratio in regions which resynthesized ATP was comparable to that in energy-depleted areas.
not appear to be the factor limiting restitution of high energy phosphates.

Resynthesis of ATP depends not only on the delivery of $O_2$, but also on the generation of reducing equivalents (NADH) for reoxidation by the electron transport chain (fig. 6). If the rate of formation of NADH in the citric acid cycle is compromised, then the production of high energy phosphates will be decreased regardless of the supply of $O_2$. In the present investigation, the generation of NADH during transient anoxia never returned to normal during recirculation; indeed, the steady decline of the anoxic-NADH transient indicates that the ability to generate NADH becomes progressively compromised during the posts ischemic period.

The marked decrement of tissue NAD$^+$ levels is further evidence of a serious disturbance of NAD$^+$-linked redox reactions. A decrease in cerebral NAD$^+$ levels following ischemia has not been reported previously; however, a nutritional deficiency of nicotinic acid (Pellagra) has been shown to cause a loss of 30-40% of NAD$^+$ in rat brain and was accompanied by profound neurologic dysfunction.$^{24}$ Thus, the 43% decline of tissue NAD$^+$ observed in the present study may severely inhibit the rate of NADH generation in the citric acid cycle and, thus, limit posts ischemic resynthesis of ATP. Evidence indicating impaired production of reducing equivalents has been detected in synaptosomes derived from hypoxic rat brain.$^{25}$ Furthermore, decreased respiratory activity of isolated mitochondria has been demonstrated following 30 minutes of incomplete ischemia in rat brain.$^{28}$

Loss of NAD$^+$ from the tissue may occur by three routes. First, NAD$^+$ may be catabolized by the enzyme, NADase, which is present in high activity in brain, and which rapidly degrades NAD$^+$ in cerebral homogenates.$^{27,28}$ Secondly, a decrease in the NAD$^+$ pool may result from the acid-catalyzed destruction of NADH,$^{29}$ the rate of which may be accelerated by ischemic acidosis. At pH 6, NADH is destroyed at the rate of approximately 20% per hour. However, assuming tissue NADH levels were maintained at 20 $\mu$mol/kg, the loss at pH 6 would be only 4 $\mu$mol/kg per hour and could not account for the entire decre-

### Table 4. Cortical Alterations during Postischemic Recirculation

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>Lac</th>
<th>NADH</th>
<th>NAD$^+$</th>
<th>NADH/NAD$^+$</th>
<th>K$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control animals:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP 2.22 $\pm$ 0.07</td>
<td>2.2</td>
<td>16</td>
<td>16</td>
<td>0.053</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Recirculated animals:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regions with ATP $&gt; 1.3$ mmol/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.62^*$ $\pm 0.02$</td>
<td>10.7*</td>
<td>21*</td>
<td>244*</td>
<td>0.086*</td>
<td>106*</td>
<td></td>
</tr>
<tr>
<td>(75)</td>
<td>(75)</td>
<td>(45)</td>
<td>(48)</td>
<td>(45)</td>
<td>(27)</td>
<td></td>
</tr>
<tr>
<td>Regions with ATP $&lt; 0.5$ mmol/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.12^*$ $\pm 0.02$</td>
<td>38.4*</td>
<td>14</td>
<td>171*</td>
<td>0.082*</td>
<td>69*</td>
<td></td>
</tr>
<tr>
<td>(67)</td>
<td>(67)</td>
<td>(46)</td>
<td>(45)</td>
<td>(45)</td>
<td>(21)</td>
<td></td>
</tr>
</tbody>
</table>

Measurements from brain recirculated for 2-4 hours.
Value are means $\pm$ SEM.
Number of samples in parentheses.
Number of animals: 3 control, 35 recirculated.
Values for ATP, Lac (lactate), and K$^+$ expressed as mmol/kg.
Values for NADH and NAD$^+$ expressed as $\mu$mol/kg.
*Different from control, $p < 0.05$. 

Figure 5. Frequency Histogram of the Number of Samples with a Given Level of ATP in Recirculated Cortex. A total of 175 samples were analyzed for ATP in 35 recirculated brains.
ment in the NAD pool. Finally, NAD+ may simply be washed out of the tissue during postischemic recirculation. Since plasma and mitochondrial membranes are normally impermeable to the pyridine nucleotides, a drastic increase of membrane permeability would have to occur to account for a major efflux of NAD+.

Tissue K+, which is primarily intracellular, was also decreased in regions with energy failure. Previously, K+ content has not been measured in small regions of brain, nor has K+ been correlated directly with metabolite levels determined in the same regional samples. A decrease of the K+/Na+ ratio was observed in macrosamples of cortical gray matter following 60 minutes of complete cerebral ischemia in the monkey. Extracellular K+ activity, measured with K+-sensitive microelectrodes, increases sharply during ischemia31 or hypoxia32 but returns to control during postischemic recirculation. Normalization of extracellular K+ can occur either by active cellular uptake or by clearance from the tissue. The present results demonstrate that regions which resynthesized ATP were able to maintain high levels of K+ content, presumably by active transport into the intracellular space. By contrast, the greatest efflux of tissue K+ occurred in areas which failed to reconstitute ATP.

The regional variations of metabolic recovery, and the factors responsible for the heterogeneity, remain perplexing. Brain regions with permanent energy failure were acidic as evidenced by decreased umbelliferone fluorescence and by increased levels of tissue lactate. The deleterious effect of excessive lactic acidosis has been noted previously3, 8, 9 and it is likely that NADH generation and energy resynthesis are directly inhibited by decreased pH. However, as in earlier reports,8, 9 end-insult levels of lactate were comparable in all areas of cortex analyzed; thus, there was no apparent regional predisposition for energy failure at the onset of recirculation.

The complexity of regional metabolic restitution, as demonstrated by the topographic techniques, emphasizes the necessity for regional measurements. Although the topographic methods used in the present study were sensitive indicators of regional abnormalities, quantitative inferences must be made with caution. Nonspecific changes, such as altered binding of NADH to tissue enzymes, might significantly influence fluorescence intensity. In particular, postischemic elevation of NADH tissue fluorescence in frozen brain did not correspond to increased levels of NADH, measured quantitatively with specific, enzymatic techniques. Thus, while the origin of the intense tissue fluorescence is not known, degradation products of NAD+, some of which fluoresce strongly, might account for the marked increase of tissue fluorescence. Whatever the explanation, it is imperative that qualitative mapping methods be validated by quantitative assay.

Although the present study has focused on "irreversible depletion" of ATP, a state representing the initial stages of cerebral infarction, regions which resynthesized ATP to greater than 1.3 mmol/kg cannot be presumed to have escaped permanent injury. Previous reports have demonstrated a severe degree of selective neuronal pathology in tissue with major restitution of energy metabolites34-36. In the present model of ischemia, there were only a few regions of cortex in which complete normalization of metabolite levels occurred. In areas with substantial, but incomplete, restoration of ATP, the persistent elevation of lactate and NADH may correspond to underlying defects which do not involve a gross depletion of energy metabolites. Indeed, complete restitution of ATP in these areas may be restricted by the decreased size of the adenylate pool38 and not be a defect in oxidative phosphorylation.

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References
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