Pathophysiology of Ischemic Cell Death:  
I. Time of Onset of Irreversible Damage;  
Importance of the Different Components  
of the Ischemic Insult  

ADELBERT AMES III, M.D., AND FRANCES B. NESBETT, A.M.

SUMMARY  Rabbit retina was used as an example of organized central nervous tissue in in vitro experiments designed to characterize the onset of cell death from ischemia. Retinas were subjected to progressively longer periods of different types of ischemic insult and then given an opportunity to recover before being tested for irreversible damage, using failure to reinstitute protein synthesis as the principal criterion. Anoxia was more damaging than substrate deprivation, but they were synergistic in combination. Restricting the volume of extracellular fluid during the combined deprivation, to simulate complete circulatory arrest in vivo, caused irreversible damage to occur even sooner. The cells were able to recover from 20 min of the complete ischemia, but it took them more than 2 h to do so. After 30 min, there was extensive irreversible damage. Loss of viability was usually associated with failure to reinstitute energy metabolism, as assessed by 2-deoxyglucose uptake. Under some circumstances loss of viability may have been the consequence of the failed energy metabolism. Increasing medium Mg++, prior to ischemia, to levels that greatly reduce energy requirements caused a significant improvement in the recovery of 2-deoxyglucose uptake.

A TEMPORARY ARREST of the circulation affects CNS cells directly by preventing exchange of substrates and products of metabolism with the circulating blood. But there are also indirect effects caused by the failure of physiological support mechanisms. These changes persist for some time after circulation is restored and may play an important role in limiting recovery. Some of the indirect effects are systemic, including failure of the respiratory center, failure of the vasomotor center, lactic acidosis, and other alterations in blood composition. Some are more localized, including loss of autoregulation and of other mechanisms for controlling flow distribution, impaired perfusion due to stasis-induced increase in blood viscosity, breakdown of the blood brain barrier, vasogenic edema, and increased intracranial pressure. In the aggregate, the direct and indirect effects of ischemia are so complex that it has been difficult to define causal sequences and to identify the time of onset of irreversible damage and the events responsible for it.

The experiments described here were designed to isolate one aspect of the overall problem for more controlled study — the response of the CNS cells themselves to the direct effects of circulatory arrest. Toward this end, we have used an isolated preparation of CNS and have subjected it in vitro to conditions that simulate an arrest of circulation in vivo. The study was concerned primarily with the problem of irreversible damage, so the experimental tissue was usually returned to the control medium for many hours following the ischemic insult before the cells' recovery was assessed by measuring their ability to reinstate protein synthesis.

For an in vitro study to be readily interpretable, it is necessary to have an experimental preparation that can be maintained in a physiological state under control conditions, and retina appears almost uniquely suited for meeting this requirement. It is very thin but strong enough to be isolated without damage; and in rabbit (as in some other species) it has no penetrating vessels but is normally nurtured by diffusion from capillaries on the surface, so it is not much disturbed by being removed from the eye to a suitable incubation medium. Retina is quite representative of other portions of the CNS with respect to its embryology, morphology, chemistry, and function; and retinal neurons and glia show the same distinctive morphological responses to various types of ischemic insults as the neurons and glia in other portions of the CNS.

The measurement of protein synthesis provides a quantitative assessment of a complex metabolic function, and offers a relatively easy method of monitoring the cells' ability to recover from an experimental insult. It has been previously shown that retinas incubating under control conditions maintain a rapid rate of protein turnover (0.5% per h), with synthesis approximately equal to breakdown. Though many aspects of CNS metabolism show large variations associated with changes in neuronal activity, protein synthesis remains remarkably constant, and this stability makes it a particularly useful measure of the basic metabolic capability of the tissue.

Some of the experiments to be described were designed to determine the maximum period of complete ischemia to which the cells can be subjected and still recover, and to determine how long their recovery takes. Other experiments were designed to examine the effect on the cells of the different components of the ischemic insult. Thus, anoxia, substrate deprivation,
and a reduction in the effective volume of extracellular fluid were presented separately or in combination to see which were the most damaging. Measurements of 2-deoxyglucose (2-DG) uptake were made following ischemia to determine whether recovery was limited by the cells' ability to reestablish their energy metabolism.

The same experimental system has been used for two additional studies, as described in the accompanying papers. One study examined the changes in plasma membrane permeability and cell volume during ischemia, and the relation of these changes to ischemic cell death. The other examined the effect on cell survival of the extracellular changes that occur during ischemia, over and above the depletion of \( O_2 \) and substrate.

It is of course hazardous to generalize from the experiments described here to conditions observed clinically. In order to make such a translation, it will be necessary to have a more complete understanding than is now available of the similarities and differences between the two situations.

The most important difference probably relates to the complex alterations that regularly occur \textit{in vivo} in both the quality and the quantity of the blood supply to the cells at risk. There are undoubtedly also important differences in the responses of the different cells involved, but it would appear likely that the differences of the cell types may be as great \textit{within} the various regions of CNS as \textit{between} regions, or even between species. The fundamental question of the molecular changes that determine death at the cellular level is still far from answered, and the similarities in this regard between all cell types probably outnumber the differences.

**Methods**

Isolation of Retina and Maintenance \textit{In Vitro}

The isolation and incubation were performed under usual laboratory illumination in a thermostatically controlled room, maintained at 37°C. New Zealand White rabbits weighing 2–2.5 kg were sedated with intravenous pentobarbitone (10 mg/kg) and brought to surgical anesthetic level with ether by mask. The eye was enucleated and hemisected, and the anterior portion and vitreous were discarded. The posterior portion was evverted under medium; the retina was separated from the choroid; and the choroid and sclera were cut from around the optic nerve. The nerve stump served as a handle for transferring the tissue but was cut off before the retina was analyzed. Anesthetics are effectively cleared from the retina by elution into the relatively large volume of medium that is used for the isolation, and electrophysiological recordings have revealed no evidence of residual anesthetic in the isolated tissue.

The incubations were performed in 6 ml or 20 ml of medium contained in a glass incubation chamber with controlled gas phase. The chamber was rocked gently in a water bath, maintained at 37 ± 0.1°C with a Lauda constant temperature circulator. The control medium was made up to resemble human CSF with respect to its electrolyte composition and its 38 organic constituents which included amino acids, vitamins, purines, pyrimidines, pyruvate, glucose (6 mmol/l), and 0.5% serum. It was equilibrated with a gas mixture of 40% \( O_2 \), 5% \( CO_2 \), and 55% \( N_2 \), saturated with water vapor.

A more complete account has recently been published of the procedure used to isolate and incubate the retina, with a detailed description of the composition and preparation of the medium and a photograph of the incubation chamber.

**Simulation of Ischemia \textit{In Vitro}**

To simulate complete circulatory arrest, the retina was immersed for 1 min in medium containing only the inorganic electrolytes equilibrated with 95% \( N_2 \) and 5% \( CO_2 \) and it was then transferred, in a nitrogen atmosphere, to a small Teflon container (fig. 1). The container was sealed and maintained in the nitrogen atmosphere at 37 ± 0.2°C for the designated period of ischemia after which the lid was removed and the retina was transferred back to the control medium.

To test the effect of anoxia alone, the retina was incubated for the specified period in medium containing the full complement of organic constituents but equilibrated with 95% \( N_2 \) and 5% \( CO_2 \). For the experiments on substrate deprivation alone, the medium contained only the inorganic electrolytes and was equilibrated with the control gas mixture of 40% \( O_2 \), 5% \( CO_2 \), and 55% \( N_2 \). And for the combined deprivation of \( O_2 \) and substrate, with a large volume of extracellular fluid, the retina was incubated in the electrolyte solu-
tion equilibrated with 95% N₂ and 5% CO₂. During these test incubations the retina was immersed in 20 ml of medium in an incubation boat that rocked gently in the water bath at 37 ± 0.1°C.

Analytic Procedures

The rate of protein synthesis was estimated from the incorporation of labeled leucine into protein. For this purpose the 11 μmole/l of leucine in the medium was enriched with L-4,5(n)-3-H leucine to a specific activity of 90 Ci/mol. The rate of glucose utilization was estimated from the uptake of 14C 2-deoxyglucose (2-DG), added to the medium at a concentration of 0.6 μmol/l with a specific activity of 330 Ci/mol. The concentration ratio of 2-DG to glucose was 1:10,000. Many of the experiments involved double labeling, and the retina was exposed to the two labeled substrates together for a ½ h period ending 15 min before harvesting. Measurements of the flux of leucine and of 2-DG (Ames, unpublished) across retinal plasma membranes have shown that the 15 min "chase" is more than sufficient to clear the tissue of unincorporated leucine and unphosphorylated 2-DG. And the labeled leucine remaining after the "chase" has corresponded closely with the TCA precipitable label.

At the time of harvest, the retina was placed in a weighing bottle and lyophilized to obtain dry weight. It was then solubilized by adding 225 μl of water followed after 45 min by 1 ml of Nuclear-Chicago Solubilizer (NCS), with mixing by magnetic stirrer beginning 20 min after adding the water. The resulting clear, colorless solution was differentially counted in a liquid scintillation counter, together with a sample of the labeling medium which had been prepared in the same way. Quench corrections did not differ between specimens and cancelled in the calculations.

Experimental Format

The retinas were kept in the control medium for 45 min to permit recovery from the effects of the isolation. Control retinas continued in the control medium for periods of up to 4 h. Test retinas were subjected to one of the 4 types of ischemic insult described above for defined periods of from 10 to 50 min and were then returned to the control medium. In experiments with the shortest recovery period, the retinas were transferred directly from the ischemic condition to control medium containing labeled leucine and 2-DG; and the results of the uptake measurements were plotted as of the midpoint of the labeling period, or 15 min along the recovery curve. In the experiments with the longest recovery period, the retinas had been back in the control medium for 4 h before being exposed to the labeled substrates.

Results

Time of Onset of Irreversible Damage

A series of experiments was performed in which retinas were exposed for successively longer periods to conditions simulating circulatory arrest in vivo. The reversibility of the ischemic damage was assessed by monitoring the retinas' ability to reinitiate protein synthesis after return to control medium (fig. 2). The ischemia caused a prompt and progressive impairment of protein synthesis, as shown by the dotted line in figure 2. Retinas that had been subjected to 20 min of ischemia showed a reduction in synthesis to 33% of the normal rate, but they were able to recover completely over a period of 2 to 4 h. Extending the duration of the ischemia to 30 min resulted in a marked change in the tissue's response. Protein synthesis appeared at first to recover quite rapidly; but recovery stopped after 2 hours, and the rate of synthesis measured at the end of 4½ h was less than 50% of control. Following 40 min of ischemia, there was a further reduction in synthetic capacity with even less recovery.

The rate at which leucine was incorporated into protein by the control retinas in figure 2 (21 nmole/g/min), corresponds to a turnover rate for total retinal protein of 0.5% per h (calculated using a value of 369 μmole per g dry wt for the leucine in retinal protein and a factor of 0.7 for the reduction in specific activity of free leucine in the intracellular pool). The change in the physical characteristics of the retina

The transition from reversible to irreversible dam-
age was associated with another, quite unexpected, change. Retinas that had been ischemic for 20 min remained flexible, and were redeployed easily when returned to the control medium; but retinas that had been ischemic for 30 min or more were quite rigid when they were removed from the Teflon container in which they had been subjected to the ischemic insult, and could be redeployed only with some difficulty when they were restored to the control medium. This change in the physical characteristics of the retina was easy to detect and was closely correlated with other indices of irreversible damage. The stiffness appears not to have been secondary to turgor from cell swelling since there was no increase in intracellular water at the time the rigidity appeared, and increasing ECF osmolarity with 29 milliosmolar polyethylene glycol beginning 4 min before the ischemia provided no protection (3 retinas). It therefore seems more likely that the rigidity reflected a change in the cytoskeleton of the cells, or a change in their plasma membranes that facilitated cross-linking between cells.

**Importance of the Different Components of the Ischemic Insult**

In order to determine which aspects of the ischemic insult were responsible for the damage that was observed after 30 min of complete ischemia, retinas were exposed for periods of 30 min to the different components, presented singly or in combination. The retinas were then returned to the control medium, and recovery was assessed by measuring their ability to reinitiate protein synthesis. The results are shown by the solid lines in figure 3. Deprivation of substrate alone was least damaging and was followed by complete recovery. Anoxia alone was followed by a 50% reduction in synthesis, but most of this was reversible, and recovery. Anoxia alone was followed by a 50% reduction in protein synthesis. The results are shown by the lowermost curve in figure 4. When substrate deprivation was added to the anoxia, there was a significant increase over the damage caused by anoxia alone. As before, reduction in ECF volume in the presence of the combined deprivation caused a further reduction in synthetic capacity, as shown by the lowermost curve in figure 4.

The uptake of 2-DG was measured in the same experiments and the results are shown in figure 5. Neither the removal of O₂ nor of substrate, alone, even when continued for as long as 50 min, left any residual deficit in 2-DG uptake; but there was a marked synergistic effect when they were removed together. The reduction in 2-DG uptake that was measured 4 h after the combined deprivation of O₂ and substrate was linearly related to the duration of the deprivation, so that there was an additional 22% reduction in 2-DG uptake combined deprivation caused a still further reduction in synthesis and a considerable increase in residual damage.

Rates of 2-DG uptake, measured in the same experiments (see interrupted lines in fig. 3), showed a rough parallelism with the measurements of protein synthesis, though 2-DG uptake was less affected than protein synthesis by deprivation of O₂ alone and by restricting the volume of ECF. As shown in the first panel of figure 2, there was a surprisingly large, though temporary, reduction in 2-DG uptake following substrate deprivation alone.

In another series of experiments to examine the effects of the different components of ischemia, the duration of the insult was varied from 30 to 50 min and recovery was not assessed until after the retinas had been back in the control medium for 4 h. When reinitiation of protein synthesis was the criterion used to assess recovery (fig. 4), the retinas showed the same rank ordering for the 4 types of ischemic insult as in figure 3. Substrate deprivation alone left no impairment of synthesis, even if continued for as long as 50 min. Anoxia alone caused a small amount of irreversible damage after 30 min (p < 0.05), that increased progressively as the duration of anoxia was extended. When substrate deprivation was added to the anoxia, there was a significant increase over the damage caused by anoxia alone. As before, reduction in ECF volume in the presence of the combined deprivation caused a further reduction in synthetic capacity, as shown by the lowermost curve in figure 4.

**Figure 3.** Retinas were subjected for 30 min to four different types of ischemic insult: deprivation of all organic substrates in the presence of normal O₂ (–SUB); deprivation of O₂ in the presence of normal substrates (–O₂); deprivation of both in a large volume of solution containing only electrolytes (–O₂, –SUB); deprivation of both, but with the ECF restricted to the interstitial fluid as described in Fig. 1 (–O₂, –SUB, –ECF). The retinas were then returned to control medium and tested, either immediately or after varying periods of recovery, for their ability to incorporate ⁴H-leucine into new protein (solid line) and for their ability to utilize glucose, as indicated by 2-DG uptake (interrupted line). Results are presented as percents of the control values ± SEM. Number of experiments in parentheses.
It is well known that synaptic transmission can be blocked by increasing the Mg$^{++}$/Ca$^{++}$ ratio in the ECF; and, in experiments on retina, it has been shown that increasing the Mg$^{++}$ in the medium causes a prompt but reversible cessation of both the spontaneous and light-evoked activity of retinal neurons, as monitored by electrophysiological recordings from the ganglion cells.  

In preliminary experiments to determine the reduction in energy requirements associated with the synaptic blockade, measurements were made of 2-DG uptake before and after increasing medium Mg$^{++}$ to 15 mmole/l in the presence of normal $O_2$ and substrates. The increase in Mg$^{++}$ caused a prompt reduction in 2-DG uptake to 32 ± 3% (S.E.M.) of the control value ($p < 0.001$, $n = 4$), and the 2-DG uptake returned promptly to normal when Mg$^{++}$ was returned to the control level of 1.2 mmole/l.

In order to see whether this reduction in their energy requirements would permit the retinas to withstand a longer period of ischemia, Mg$^{++}$ was increased to 15 mmole/l beginning 4 min before a 30 min deprivation of $O_2$ and substrate in a restricted volume of ECF. The Mg$^{++}$ was kept elevated through the first hour of recovery and the retinas were assessed after 4 h of recovery (table 1). Those that had had an elevated ECF Mg$^{++}$ during the ischemic period showed only a 12% reduction in 2-DG uptake whereas the retinas that had been made ischemic with normal levels of Mg$^{++}$ showed a 36% reduction in 2-DG uptake ($p < 0.005$). The elevated Mg$^{++}$ provided no significant protection with respect to protein synthesis, and a possible explanation for this disparity is presented below.

**Reproducibility of the Experimental System**

As indicated by the error bars in the figures, repli-
cate retinas were quite similar in their responses, and this was true even for tissues that had been severely damaged by the ischemic insults. In 8 groups of replicates in which the retinas had not been significantly impaired by the ischemic insult, the standard deviation averaged 6% of the mean for leucine incorporation and 8% for 2-DG uptake. In 18 groups in which the retinas had been significantly damaged, the standard deviations for leucine incorporation and 2-DG uptake averaged 7% and 5% of the normal means respectively.

Discussion

Time of Onset of Irreversible Damage

When retinas were returned to control medium after 20 min of ischemia, protein synthesis recovered fully over a period of 4 h (fig. 2). It can probably be inferred from this that the cells had also recovered their ability to synthesize new messenger RNA; since, in the absence of synthesis, the normal degradation rate of mRNA would be expected to have led to a significant reduction in its concentration over the 4 h period. The resumption of transcription and translation indicates that a major portion, and a complex portion, of the cells' metabolism was again operating normally; and it seems reasonable to conclude from this that the cells had remained viable.

On the other hand, retinas that had been subjected to 30 min of ischemia recovered less than 50% of their protein synthetic capacity; and the recovery that did occur took place in the first 2 h (fig. 2), suggesting that a longer period of recovery would not have resulted in further improvement. This conclusion receives additional support from other experiments in which changes in intracellular water were measured following 30 min of ischemia and in which it was found that, as the recovery time was extended, the cells continued swelling rather than returning towards normal size.

Thus the results of these experiments indicate that an important change occurred between 20 and 30 min of ischemia, resulting in the irreversible loss of more than 50% of the retina's protein synthetic capacity and probably reflecting the loss of viability of a considerable fraction of the retinal cells. The appearance of irreversible damage between 20 and 30 min of ischemia is consistent with the results of previous studies in which changes in intracellular water were measured following 30 min of ischemia and in which it was found that, as the recovery time was extended, the cells continued swelling rather than returning towards normal size.

Thus the results of these experiments indicate that an important change occurred between 20 and 30 min of ischemia, resulting in the irreversible loss of more than 50% of the retina's protein synthetic capacity and probably reflecting the loss of viability of a considerable fraction of the retinal cells. The appearance of irreversible damage between 20 and 30 min of ischemia is consistent with the results of previous studies in which changes in intracellular water were measured following 30 min of ischemia and in which it was found that, as the recovery time was extended, the cells continued swelling rather than returning towards normal size.

A 20 to 30 min survival time for CNS cells subjected to complete ischemia is longer than would have been expected from most clinical observations or from most experimental studies performed in vivo. Circulatory arrest usually leads to irreversible loss of CNS function after 5 min, and it has been generally accepted that this is the time at which the more vulnerable cells are beginning to undergo irreversible damage. However, it is becoming increasingly apparent that recovery from ischemia in vivo may be very much curtailed by the unphysiological conditions to which the CNS is subjected in the period following the ischemia. When unusual care has been taken in the post-ischemic peri-
Energy Metabolism and Irreversible Damage

Failure of energy metabolism clearly plays a primary role in the changes that occur during the ischemia and must be an important causal link in the chain leading to irreversible damage, but there is still considerable uncertainty as to whether a failure to recover energy metabolism is a manifestation of the earliest irreversible damage, and whether, indeed, the failure to recover energy metabolism is itself the cause of the loss of viability.\(^{24}\) The measurement of a reduction in energy metabolism in post-ischemic CNS tissue is open to some ambiguity of interpretation, since it might reflect failure of the tissue to resume its physiological activities, rather than a primary failure of the reactions responsible for the generation of metabolically usable energy. In the experiments reported here, deprivation of \(O_2\) and substrate led to an irreversible reduction in 2-DG uptake after 30 min, with a linear progression of the deficit as the deprivation was extended to 40 and 50 min. Since electrophysiological studies have shown virtually no functional recovery after 30 min of deprivation,\(^2\) this progressive reduction in glucose utilization is probably not secondary to a progressive failure of physiological function.

The metabolic consequences of anoxia have little in common with those of substrate deprivation, except for the reduction in ATP synthesis. Therefore the strikingly synergistic effect of the combined deprivation on the recovery of 2-DG uptake (fig. 5) suggests that the failure to reestablish glucose metabolism depended on the extent of ATP depletion during the deprivation. It apparently made little difference whether the ATP was being maintained by oxidative metabolism or glycolysis. The results are consistent with the hypothesis that the cells lost their ability to reestablish glucose metabolism when the ATP fell below a critical level, determined perhaps by the \(K_m\) with hexokinase. However, it is also possible that the failure of glucose metabolism was secondary to other changes in the cells' metabolism resulting from the ATP depletion.

If irreversible damage to glucose metabolism depended directly on the extent of ATP depletion, measures to reduce the requirements for ATP should be protective. This probably accounts for the improved recovery when \(Mg^{++}\) was increased prior to ischemia to a level that blocks synaptic transmission (table 1). Alternative explanations for the protective effect of \(Mg^{++}\) include stabilization of plasma membranes\(^{25}\) and reduction in the flux of \(Ca^{++}\) into the ischemic cells.\(^{26}\) The observation that elevated \(Mg^{++}\) improved the recovery of glucose metabolism more than protein synthesis (table 1) may be explained by the fact that a large proportion of retinal glycolysis takes place in the inner retina,\(^{20}\) whereas a large proportion of the protein synthesis takes place in the photoreceptor cells, which would not have been affected by synaptic blockade.

The above considerations suggest that failure to reestablish energy metabolism may have been the cause of the loss of viability following deprivation of \(O_2\) and substrate in a large volume of ECF. Retinas subjected to this condition showed a close correlation between failure to recover 2-DG uptake and failure to recover protein synthesis (see fig. 3 and figs. 4 and 5); and there were no instances in which the retinas recovered a normal rate of 2-DG uptake while exhibiting a continued impairment of protein synthesis as would have been expected if early irreversible changes had interfered directly with transcription and translation.

However, failure to reestablish glycolysis does not appear to be a sufficient explanation for loss of viability following other types of ischemic insult. When ECF volume was reduced during the deprivation to simulate complete circulatory arrest, there was a marked increase in irreversible damage as assessed by protein synthesis, without any further reduction in glucose uptake (cf. figs. 4 and 5). These retinas also exhibited a striking loss of flexibility at about the time of irreversible damage; and, in retinas that were simi-
larly treated, we have observed a progressive increase in plasma membrane permeability. These findings suggest that, under the conditions of complete circulatory arrest, there are early irreversible changes involving systems other than those concerned with energy metabolism.

Following anoxia alone, there was an irreversible reduction in protein synthesis (fig. 4) before there was a measurable effect on glucose utilization (fig. 5). O₂ uptake was not measured in the post-ischemic retinas, and it is possible that there was a failure of oxidative energy metabolism in the mitochondria-rich photoreceptors that was not reflected in the measurement of 2-DG uptake. Mitochondria have been identified as an early site of ischemic damage.²⁷

A conclusive answer to the question of whether failure to reinstitute energy metabolism is the limiting factor in survival following some types of ischemic insult will probably depend on devising a way of circumventing the problem and then seeing whether this permits recovery of the rest of the cells' metabolic processes. Experiments of this type have been performed on red cells that had been subjected to red cell storage disease and that were no longer able to utilize glucose. Administration of inosine made it possible for them to initiate glycolysis, and to recover normal ion gradients and a normal life-span.²⁸

References
Pathophysiology of ischemic cell death: I. Time of onset of irreversible damage; importance of the different components of the ischemic insult.
A Ames, 3rd and F B Nesbett

Stroke. 1983;14:219-226
doi: 10.1161/01.STR.14.2.219

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/14/2/219

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at: http://stroke.ahajournals.org/subscriptions/