Effect of Anoxia on Protein Metabolism in Subcellular Fractions of Rabbit Brain

BY TAKEHIKO YANAGIHARA, M.D.

Abstract:
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The effect of cerebral anoxia on leucine incorporation into proteins of various subcellular fractions from rabbit brain was studied by using a previously reported in vitro model. Although the decrease in leucine incorporation was observed earlier in nuclei and microsomes, other fractions also demonstrated a rapid decrease. These results were quite different from the pattern of leucine incorporation observed in a type of reversible suppression produced with diphenylhydantoin.

Additional Key Words
leucine incorporation brain slices in vitro model

In previous communications, an in vitro experimental model for cerebral anoxia was presented. This model made available homogeneous anoxic brain tissue for the investigation of macromolecular metabolism at the cellular and subcellular levels. Although there was decreased amino acid incorporation into proteins during early recovery after short anoxic periods, the recovery was complete within 60 minutes. On the other hand, only little tendency of recovery was observed after long anoxic periods.

In the present report, the investigation was extended to study amino acid incorporation into proteins of various subcellular fractions during the early recovery stage (30 minutes) to see if any particular subcellular fraction is responsible for the above-mentioned decrease in amino acid incorporation. The pattern of amino acid incorporation was compared with previously available data on reversible neurosuppression produced by diphenylhydantoin, since there was selective inhibition of the nuclear fraction during the early stage in that study.

Methods
The preparation of tissue slices and the method for study of amino acid incorporation during incubation are based on the previously reported technique with minor modification.

Albino rabbits (2.5 to 3.0 kg) were lightly anesthetized by intravenous injection of pentobarbital and were rapidly perfused with ice-cold Ringer's solution through the left ventricle of the heart. Each brain was quickly removed and placed in crushed ice. Each cerebral hemisphere was then sliced with a McIlwain tissue chopper at 0.2 or 0.4 mm thick, and approximately 4.0 gm of tissue were transferred to a 125-ml Erlenmeyer flask containing 20 ml of incubation medium containing tris buffer, electrolytes, and glucose, as described previously. Each flask was capped with a rubber stopper carrying two glass tubes and preincubated for 15 minutes at 37°C as described previously. At the end of the preincubation period, the tip of the input glass tube was lowered into the incubation medium; the flask for the experimental group was gassed with 100% nitrogen while the flask for the control group was gassed with oxygen. The tip of the glass tube was maintained below the surface of the incubation medium for two minutes and then elevated to the previous level, and incubation was continued for the prescheduled anoxic period for the experimental group (5 to 30 minutes).

At the end of this incubation period, brain slices from each flask were recovered by sieving and transferred to another 125-ml Erlenmeyer flask containing the same incubation medium except for addition of l-[4,5^-H]leucine (Amersham/Searle; adjusted to 100 mCi/mmol) at a concentration of 5 μCi/ml. The flask was capped in the same way and incubation was continued for 30 more minutes under constant oxygen flow.

At the end of incubation, one or two tissue slices were taken for the determination of acid-soluble radioactivity and the rest were briefly centrifuged to recover the tissue as a pellet. Subsequent procedures were carried out at 4°C. The pellet was resuspended in 0.32 M sucrose in 10 mM tris-HCl buffer, pH 7.4 (standard sucrose solution). The preparation of various subcellular fractions was similar to what has previously been described except for the myelin fraction.

Homogenization was carried out with ten up-and-down strokes in a loosely fitted Teflon-glass homogenizer driven at 800 rpm. A sample was taken for the determination of acid-soluble radioactivity and the rest were centrifuged to recover the tissue as a pellet. Subsequent procedures were carried out at 4°C. The pellet was resuspended in 0.32 M sucrose in 10 mM tris-HCl buffer, pH 7.4 (standard sucrose solution). The preparation of various subcellular fractions was similar to what has previously been described except for the myelin fraction.

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the same speed and the resulting supernatant fraction was further centrifuged at 12,000 × g for 30 minutes to pellet the crude mitochondria. This pellet was resuspended in standard sucrose solution, layered on top of a discontinuous gradient consisting of 0.8 and 1.2 M sucrose, and centrifuged at 55,000 × g for 90 minutes. The “mitochondria” fraction was recovered as a pellet, the “synaptosomes” fraction was aspirated from the 0.8 and 1.2 M sucrose, and the “myelin” fraction was aspirated from the 0.32 and 0.8 M sucrose. Synaptosomes and myelin were diluted with 40 ml of standard sucrose solution and recovered by centrifugation at 12,000 × g for ten minutes. The supernatant fraction from the crude mitochondria was further centrifuged at 150,000 × g for 60 minutes to separate microsomes (as a pellet) and the soluble fraction.

Each particulate fraction was homogenized in standard sucrose solution and precipitated with an equal volume of cold 10% trichloroacetic acid (TCA). Each precipitate was washed three times with cold 5% TCA (the third wash being heated at 90°C for 15 minutes), extracted with alcohol-ether (1:1), and dried with ether. The dried sample was dissolved in 1.0 N NaOH and an aliquot was taken for protein determination according to Lowry et al. Another aliquot was solubilized with Soluene-100 (Packard Instrument Co.) and counted in toluene-based scintillation solution in a Nuclear-Chicago Mark II liquid scintillation spectrometer. Quench correction was calculated from an external standard ratio integrations per minute (dpm) per milligram of protein.

Brain slices taken at the end of the incubation procedure were weighed and homogenized in 1.0 ml of cold 5% TCA. To determine the acid-soluble radioactivity derived from [3H]leucine, the precipitate was removed by centrifugation, and an aliquot of the supernatant fraction was dissolved in Insta-Gel (Packard Instrument Co.) and counted in the same way as for protein radioactivity. The specific radioactivity was expressed as dpm per milligram of wet tissue.

Results
The specific radioactivities of proteins from each subcellular fraction are shown in table 1, from five independent experiments with normal brain slices. Leucine incorporation was high in microsomes and nuclei and lower in myelin and synaptosomes. The pattern is a little different from that previously reported with rat brain or rabbit brain during short periods of incorporation and single-stage incubation, in which very high values were observed in nuclei. However, the pattern in the present series is somewhat similar to the pattern for in vivo incorporation with rabbit brain except for very high specific radioactivity in microsomes. These differences will be discussed later.

The specific activities in anoxic tissue were calculated as percentages of those in the control group that was processed simultaneously with the anoxic group. Although higher incorporation was observed with brain slices 0.2 mm thick, the specific activity percentages were similar for slices 0.2 and 0.4 mm thick. There was a marked decrease in leucine incorporation with longer anoxic periods, as can be seen with the homogenate (figs. 1 and 2), and values at each anoxic period for the homogenate are similar to those during the first 30 minutes in the previous study. Although a decrease in specific radioactivity was observed in each subcellular fraction, a prompt decrease was seen in the nuclei and microsomes, as shown by the lack of a “shoulder” in the declining curve of these two fractions. The values at anoxic periods of five and seven minutes were already 60% to 70% of control values, and they rapidly decreased to

<table>
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<td>Leucine Incorporation Into Proteins of Various Subcellular Fractions</td>
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<tr>
<td>Fraction</td>
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<td>Homogenate</td>
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<td>Nuclei</td>
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<td>Mitochondria</td>
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*Results are expressed as mean ± SD for five independent experiments.

Stroke, Vol. 5, March-April 1974
Leucine incorporation into proteins of subcellular fractions after various anoxic periods, as mean (± SE) percentage of control value. The data were based on four experiments for five-minute anoxic periods, five experiments for seven minutes, six experiments for ten minutes, and four experiments for 15, 20, and 30 minutes (the numbers for the myelin fraction were one less than designated for 7, 15, and 30 minutes).

Discussion
Protein metabolism has been investigated in relation to cerebral anoxia or ischemia, as briefly summarized in the past. However, the investigation has not been well extended to the various subcellular elements that make up the brain structure, except for the case of moderate hypoxia of longer duration. Therefore, the present investigation was designed to determine if a simple subcellular fraction is responsible for the decrease of amino acid incorporation or if multiple fractions are involved simultaneously. This appeared particularly interesting because differences in response of various subcellular structures have been observed in reversible neurosuppression.

In the control group, there were some differences in the pattern of leucine incorporation in various subcellular fractions compared to previously reported data. The lower radioactivity in nuclei than in microsomes in the present study could be based on the difference between one-stage and two-stage incubation. High incorporation in the microsomal fraction may be related to the higher speed of centrifugation used to precipitate crude mitochondria in the present study, which would minimize the synaptosomal contamination of microsomes and result in a higher ratio of polysomes compared to membranous elements in the microsomal fraction. Use of the incubation medium previously used did not change these patterns.

Although the decrease in radioactivity was more diffuse, the nuclear and microsomal fractions showed quicker decreases. Because selective inhibition in the nuclear fraction was observed in the very early stage of neurosuppression produced by diphenylhydantoin, this could be a common and very early phenomenon in neurosuppression, as predicted previously. However, simultaneous decrease of radioactivities in other subcellular fractions indicates a diffuse involvement in the anoxic condition, in comparison to the more specific neurosuppression produced by pharmacological agents.

The microsomal fraction contains polysomes that are the main site of protein synthesis. The marked decrease in leucine incorporation in this fraction appears to be responsible for a rapid decrease of leucine incorporation in the brain homogenate and indicates that polypeptide synthesis in polysomes is involved very early in the anoxic condition. Such a drastic change in microsomes was not reported with prolonged moderate hypoxia and did not exist during the early stage of neurosuppression produced by diphenylhydantoin. Marked inhibition also was observed in the synaptosomal fraction. Since there has been controversy about protein synthesis in the synaptosomal element per se and since contamination from ribosomal structures appears to be inevitable, it cannot be determined whether this marked inhibition originates from the synaptosomal element or from contaminating elements. Since microsomal contamination of the synaptosome fraction has to be expected because of the crudeness of the preparation used here, a considerable portion of the radioactivity in synaptosomes should be of microsomal origin. For the same reason, the myelin fraction also would be contaminated with microsomes. The slower decrease of specific radioactivity in mitochondria is of interest because this fraction is the main site of oxidative phosphorylation and is responsible for ATP production.

Comparison with the pattern of leucine incorporation observed in reversible neurosuppression is interesting. Although inhibition of leucine incorporation was seen in all subcellular elements at the height of neurosuppression with diphenylhydantoin, there...
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was definite preferential inhibition in the nuclear fraction in the early stage. Inhibition is more diffuse in the case of anoxia, and this suggests that rapid inhibition of leucine incorporation occurs in both neuronal and neuroglial elements simultaneously in the case of cerebral anoxia.

Acknowledgments
The author is indebted to Mrs. Rita M. Seghers and Mrs. Mary C. Riewe for their careful technical assistance.

References
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Stroke. 1974;5:226-229
doi: 10.1161/01.STR.5.2.226

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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