somal DNA [Fig. 2, inset (*8*)]. The shape of each broad set of peaks is reproducible and amounts to a "fingerprint" of that particular turn of the DNA helix.

In particular, we observe that certain individual sharp peaks in the tracings for the HSV-1 tk DNA are less intense than would be expected from the intensities of nearby peaks. We find nearly all of the peaks of low intensity (representing backbone deoxyriboses of lower than expected reactivity toward hydroxyl radical) in sequences in which a pyrimidine nucleotide occurs to the 5′ side of a purine [pyrimidine(3′-5′)purine], with the purine giving rise to the peak of low intensity. An excellent example is the peak representing the guanine at position 36 in Fig. 2. Almost all pyrimidine(3′-5′)purine sequences have this characteristic. This observation recalls the analysis by Calladine (*4*) of the sequence-dependence of conformation of the dodecanucleotide d(CGCGAATTCGCG). Calladine explained variations in base pair twist angle, roll, propeller twist, and slide by showing that the steric clash of purines on opposite strands of the DNA helix could lead to perturbations in DNA structure. Pyrimidine(3′-5′)purine sequences are particularly susceptible to such steric clash and exhibit the largest deviation from the regular *B*-DNA conformation, according to Calladine's analysis. The sequence-dependence of the reactivity of hydroxyl radical toward deoxyribose residues along the helix is evidence for similar systematic variations (*4*, *5*) of the shape of the surface of DNA in solution.

One reason for studying the helical periodicities of DNA molecules that contain regulatory sequences is to determine the relative orientations of proteins that bind to such sequences. The densitometer scan in Fig. 2 shows where on the helix the transcriptional control sequences (*9*) of the HSV-1 tk gene lie. After the "footprints" (sequence preferences) (*20*) of transcription factors bound to these regulatory regions (*21*) are determined, we should be able to deduce from our data the relative orientations of these proteins as they are bound along the helix.

The method we report here offers a simple way to determine the helical twist and other structural details of any DNA restriction fragment. We hope that its application to a wide variety of DNA molecules will provide a connection between high-resolution structural studies of oligonucleotides (*1*–*5*) and the conformations of regions of DNA in their natural contexts.

References and Notes

1. R. E. Dickerson, H. R. Drew, B. N. Conner, R. M. Wing, A. V. Fratini, M. L. Kopka, *Science* **216**, 475 (1982).
2. O. Kennard, *Pure Appl. Chem.* **56**, 989 (1984).
3. D. J. Patel, A. Pardi, K. Itakura, *Science* **216**, 581 (1982).
4. C. R. Calladine, *J. Mol. Biol.* **161**, 343 (1982).
5. R. E. Dickerson, *ibid.* **166**, 419 (1983).
6. C. R. Calladine and H. R. Drew, *ibid.* **178**, 773 (1984).
7. H. R. Drew and A. A. Travers, *Cell* **37**, 491 (1984).
8. D. Rhodes and A. Klug, *Nature (London)* **286**, 573 (1980).
9. S. L. McKnight *et al.*, *Cell* **37**, 253 (1984).
10. D. Rhodes and A. Klug, *Nature (London)* **292**, 378 (1981).
11. M. Behe, S. Zimmerman, G. Felsenfeld, *ibid.* **293**, 233 (1981).
12. R. P. Hertzberg and P. B. Dervan, *J. Am. Chem. Soc.* **104**, 313 (1982).
13. C. Bull, G. J. McClune, J. A. Fee, *ibid.* **105**, 5290 (1983).
14. R. P. Hertzberg and P. B. Dervan, *Biochemistry* **23**, 3934 (1984).
15. Nucleosomal DNA was labeled at the 5′ ends by reaction with polynucleotide kinase and [γ-^{32}P]ATP. Solutions of 50 m*M* $CaCl_2$ and 80 m*M* K_2HPO_4 were prepared by dissolving $CaCl_2$ hydrate (Aldrich, Gold Label) and $K_2HPO_4 \cdot 3H_2O$ (Sigma) in 50 m*M* tris-HCl (*p*H 8.0, Sigma). Equal volumes of these solutions were mixed to generate a suspension of calcium phosphate microcrystals. Experimental samples consisted of calcium phosphate suspension, 1 μg of nonradioactive carrier nucleosomal DNA, and radioactive DNA in a total volume of 70 μl. [In samples without calcium phosphate precipitate, 50 m*M* tris-HCl (*p*H 8.0) was substituted for the calcium phosphate suspension.] DNA was allowed to bind to the precipitate for at least 1 hour before digestion. A solution of iron(II) EDTA was prepared immediately before use by mixing equal volumes of 0.2 m*M* $(NH_4)_2FeSO_4 \cdot 6\ H_2O$ (Aldrich) and 0.4 m*M* EDTA. The cutting reaction was initiated by placing iron(II) EDTA solution (10 μl), 0.3 percent H_2O_2 (10 μl, Baker), and 10 m*M* L-ascorbic acid (sodium salt) (10 μl, Sigma) on the inner wall of the 1.5-ml Eppendorf tube containing the DNA sample, allowing the reagents to mix, and then adding the reagent to the DNA-containing suspension. After the desired time the reaction was quenched by adding 0.1*M* thiourea (10 μl), 0.2*M* EDTA (32 μl), transfer RNA (5 μg), 0.3*M* sodium acetate (200 μl), and absolute ethanol (750 μl). The DNA was precipitated twice and then dissolved in a formamide-containing dye mixture. DNase I digestions (*8*) were performed for 5 minutes. Electrophoresis was performed at 1500 V on a high-bisacrylamide denaturing polyacrylamide gel (8.0 percent acrylamide, 1.2 percent *N*,*N*′-methylenebisacrylamide, 50 percent urea) prepared by the method of Lutter (*16*).
16. L. C. Lutter, *Nucleic Acids Res.* **6**, 41 (1979).
17. The 207-bp HSV-1 tk DNA restriction fragment was isolated from a clone of the gene and its upstream and downstream sequences in plasmid pBR322 (*9*). The particular clone we studied has a single mutation (G changed to C) at position −50 relative to the cap site of the tk gene (*9*). Supercoiled plasmid DNA was linearized by digestion with Bam HI, radioactively labeled at the 3′ ends by reaction with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP, and digested with Bgl II to give a 207-bp fragment labeled on the noncoding strand. The helical twist determination experiment was performed exactly as for nucleosomal DNA (*15*).
18. For DNA to adopt a particular azimuthal orientation on the inorganic surface (*8*) we find it important to use restriction enzymes that leave unpaired ends a few bases in length, in agreement with Rhodes and Klug (*8*), who reported that nucleosomal DNA, trimmed of its overhanging ends by S1 nuclease, did not orient in a particular way on the inorganic surface. Both Bgl II and Bam HI leave four-base 5′ overhangs.
19. L. J. Peck and J. C. Wang, *Nature (London)* **292**, 375 (1981).
20. M. M. Van Dyke and P. B. Dervan, *Science* **225**, 1122 (1984).
21. D. Gidoni, W. S. Dynan, R. Tjian, *Nature (London)* **312**, 409 (1984).
22. A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980).

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Hypoglycemia-Induced Neuronal Damage Prevented by an *N*-Methyl-D-Aspartate Antagonist

Abstract. *The possibility that neuronal damage due to hypoglycemia is induced by agonists acting on the* N-*methyl-*D-*aspartate (NMDA) receptor was investigated in the rat caudate nucleus. Local injections of an NMDA receptor antagonist, 2-amino-7-phosphonoheptanoic acid, were performed before induction of 30 minutes of reversible, insulin-induced, hypoglycemic coma. Neuronal necrosis in these animals after 1 week of recovery was reduced 90 percent compared to that in saline-injected animals. The results suggest that hypoglycemic neuronal damage is induced by NMDA receptor agonists, such as the excitatory amino acids or related compounds.*

TADEUSZ WIELOCH

Laboratory for Experimental Brain Research, University of Lund, S-221 85 Lund, Sweden

Excitotoxins, in particular the excitatory amino acids glutamate and aspartate, have been implicated in the pathogenesis of brain damage in various neurological diseases, including temporal lobe epilepsy, Huntington's disease, olivopontocerebellar atrophy, and cerebral ischemia (*1*). Hypoglycemia, especially if severe enough to cause coma (*2*), is yet another pathological condition leading to extensive neuronal loss in selected brain areas (*3*). The mechanisms underlying the sensitivity of neurons to glucose deprivation are not understood. However, with the advent of a long-term recovery model of insulin-induced hypoglycemia in rats (*4*), mimicking the clinically most relevant conditions of hypoglycemia—those created by an intravenous overdose of insulin—it has become possible to study these mechanisms.

The brain needs a continuous supply of oxygen and glucose for functional integrity. A progressive decrease in blood glucose concentration leads to metabolic

responses in the brain characterized by increased utilization of endogenous substrates, membrane depolarization, and energy failure (*5*). These metabolic events are accompanied by a pervasive neuronal necrosis affecting, among other structures, the caudate nucleus (*6*). Neuronal necrosis is not observed unless the electroencephalogram (EEG) shows periods of isoelectricity (*4*). This suggests that the events associated with cessation of EEG, namely energy failure and loss of ion homeostasis, may trigger deleterious events, such as excessive release of glutamate or aspartate, eventually leading to cell damage.

In this context, a subtype of glutamate receptors, the *N*-methyl-D-aspartate (NMDA) receptor, is of particular interest. Antagonists of these receptors protect neurons in several brain regions against the adverse effects of excitotoxins (*7*). For example, injections of quinolinic acid, an endogenous excitotoxin, into the striatum induced neuronal necrosis that was mitigated by simultaneous administration of the NMDA receptor antagonist 2-amino-7-phosphonoheptanoic acid (AP7) (*8*). The study reported here was performed to determine whether neuronal necrosis induced by hypoglycemia can be associated with an excitotoxin-induced phenomenon mediated by NMDA receptors.

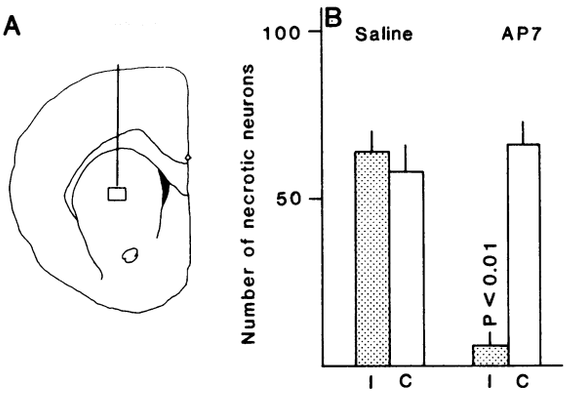

Fig. 1. (A) Diagram showing the coronal section (*21*), the approximate position of the needle track, and the area (square) where cell counts were performed. (B) Neuronal necrosis in the caudate nuclei of the control and AP7-injected groups. The total neuronal population in the investigated area was 111 ± 4 cells. I, injected hemisphere and C, contralateral hemisphere. Values are means ± standard errors.

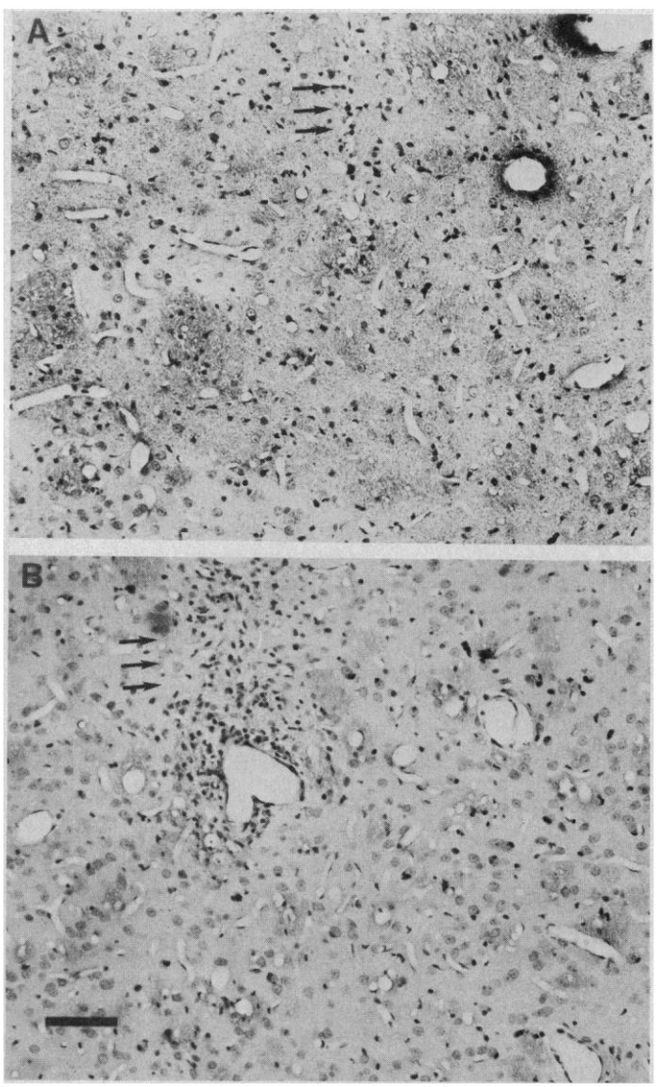

Fig. 2. (A and B) Photomicrographs of the middle portion of the caudate nucleus of rats subjected to 30 minutes of hypoglycemic coma and allowed to recover for 1 week. (A) Effect of intrastriatal injection of 2 μl of saline (needle track indicated by arrows). Necrotic neurons, composing approximately 60 percent of the total neuronal population, are indicated by arrowheads. (B) Section taken after intrastriatal injection of 40 μg of AP7 (needle track indicated by arrows). Apart from reactive gliosis at the injection site, no neuronal necrosis is visible. In both groups the needle track was identified and traced from the surface of the cortex. Scale bar, 100 μm.

Hypoglycemic coma was induced in rats by intraperitoneal injection of insulin (*9*). In experimental animals (n = 6), 40 μg of AP7 was injected into the caudate nucleus of one hemisphere, while control animals (n = 6) received an injection of saline at the same site. Both injections were made 30 to 40 minutes before the EEG became isoelectric (*10*). Animals then remained in a coma for 30 minutes, a period that reproducibly induces neuronal necrosis (60 to 80 percent) in the caudate nucleus (*4*). At the end of the 30-minute period, glucose was administered through a venous catheter and the animals were allowed to recover for 1 week. The brains were fixed by perfusion, embedded in paraffin, and sectioned, and the sections were stained with celestine blue and acid fuchsine (*11*). Neuronal necrosis was assessed in the caudate nuclei of both hemispheres by cell counting (*12*). Necrotic neurons appeared bright red with a condensed nucleus. In some neurons the nucleus was fragmented. Surviving cells were violet with a prominent round nucleus and nucleolus (*4*, *13*).

Cell counts were made in a 600 by 400 μm area in tissue sections where the needle track was widest (Fig. 1A) (*12*). In the saline-injected side of control brains, 64 ± 6 (mean ± standard error) of the 105 ± 6 neurons examined were necrotic, while on the contralateral side 58 ± 8 of 102 ± 7 neurons were damaged. In the AP7-injected side of experimental brains, 6 ± 4 of 127 ± 10 neurons were damaged, while in the contralateral hemisphere 66 ± 8 neurons of 109 ± 5 were affected. Four animals showed no neuronal necrosis. In two animals, 23 of 135 and 12 of 93 neurons were necrotic. The decrease in neuronal necrosis in the AP7-injected hemisphere was significant ($P < 0.01$) compared to the contralateral side and to both caudate nuclei of the control group (Fig. 1B) (*14*).

Figure 2A shows a photomicrograph

of the caudate nucleus of a rat injected with saline. In this animal 62 percent of the neuronal population was damaged in the injected hemisphere. Figure 2B shows the corresponding area in an animal injected with AP7. Gliosis was observed around the injection site, but no damaged neurons could be detected. In the contralateral caudate nucleus 69 percent of the neurons were necrotic.

Several observations support the idea that hypoglycemic brain damage is mediated by excitotoxins. First, at the onset of isoelectric EEG, the extracellular levels of aspartate and glutamate are markedly increased (*15*). Second, unilateral ablation of the motor cortex, transecting the corticostriatal projections and decreasing the ipsilateral striatal content of glutamate by 10 percent, protects the subjacent caudate nucleus against neuronal necrosis after 30 minutes of hypoglycemic coma (*16*). This suggests that synaptic events are important for the induction of neuronal necrosis. Furthermore, an electron microscopic investigation of the dentate gyrus granule cells (*17*), which are vulnerable to hypoglycemia (*4*), revealed a dendro-somatic, axon-sparing lesion, that is, similar ultrastructural characteristics as observed in the excitotoxin-induced neuronal damage (*18*).

As in severe hypoglycemia, cerebral ischemia leads to extensive energy deprivation, membrane depolarization, and an increase in extracellular levels of excitatory amino acids (*5, 15, 19*). Since AP7 ameliorates the acute morphological changes in the hippocampus induced by ischemia (*20*), similar pathogenic mechanisms could prevail in the two disorders. However, the distribution of neuronal necrosis after ischemia is different from that after hypoglycemia (*6, 13*), suggesting that the NMDA receptor agonists released, their origin, and their regional extracellular concentrations may differ (*15*).

These findings show that the extent of neuronal necrosis in the caudate nucleus induced by severe hypoglycemia can be significantly reduced by an NMDA receptor antagonist, suggesting that the deleterious mechanisms leading to neuronal necrosis may be mediated by excitotoxins. Excitatory amino acid antagonists may prove useful in preventing hypoglycemic brain damage.

References and Notes

1. J. T. Coyle *et al.*, *Neurosci. Res. Program Bull.* **19**, 330 (1981); J. T. Coyle and R. Schwarcz, *Nature (London)* **263**, 244 (1976); E. G. McGeer and P. L. McGeer, *ibid.*, p. 517; A. Plaitakis, S. Berl, M. D. Yahr, *Science* **216**, 193 (1982); R. P. Simon *et al.*, *ibid.* **226**, 850 (1984); T. Wieloch *et al.*, *Neurol. Res.* **7**, 24 (1985); T. Wieloch, *Prog. Brain Res.*, in press.
2. V. Marks, *Br. Med. J.* **1**, 430 (1972); A. T. Paz-Guevara, T.-H. Hsu, P. White, *Diabetes* **24**, 559 (1975); D. S. Wilkinson and L. D. Prockop, *Handb. Clin. Neurol.* **27**, 53 (1976); T. Deckert, J. E. Poulsen, M. Larsen, *Acta Med. Scand. (Suppl.)* **624**, 48 (1979).
3. J. B. Brierley and D. I. Graham, *Greenfield's Neuropathology* (Arnold, London, ed. 4, 1984), p. 150.
4. R. N. Auer, Y. Olsson, B. K. Siesjö, *Diabetes* **33**, 1090 (1984).
5. B. K. Siesjö and C.-D. Agardh, *Handbook of Neurochemistry* (Plenum, New York, ed. 2, 1983), vol. 3, p. 353; T. Wieloch *et al.*, *Neurochemistry* **43**, 160 (1984).
6. R. N. Auer *et al.*, *Acta Neuropathol.* **64**, 177 (1984).
7. J. W. Olney *et al.*, *Brain Res.* **221**, 207 (1981); J. W. Olney, *Excitotoxins* (Macmillan, London, 1983), p. 82; R. Schwarcz, J. F. Collins, D. A. Parks, *Neurosci. Lett.* **33**, 85 (1982); R. Schwarcz *et al.*, *Exp. Neurol.* **84**, 1 (1984).
8. R. Schwarcz, W. O. Whetsell, Jr., R. M. Mangano, *Science* **219**, 316 (1983); R. Schwarcz, W. O. Whetsell, Jr., A. C. Foster, *Excitotoxins* (Macmillan, London, 1983), p. 122; R. Schwarcz and C. Köhler, *Neurosci. Lett.* **38**, 85 (1983).
9. Twelve male Wistar rats (300 to 350 g) (Mollegaard Avlslaboratorium A/S) were used. Hypoglycemia was induced (*4*) and the animals were fasted overnight. After being injected with insulin (8 IU/kg, intraperitoneally; Actrapid, Novo), the rats were anesthetized, intubated, immobilized with suxamethonium chloride, and ventilated [N_2O and O_2 (70:30)] with a respirator. The EEG was continuously monitored with two subcutaneous needle electrodes. Mean arterial blood pressure was recorded through a tail artery catheter, and blood gases (pO_2 and pCO_2) and *p*H were intermittently monitored. The animals were kept at 37°C with a heating bulb. After 30 minutes of isoelectric EEG, the animals were given a bolus injection of 50 percent glucose (0.5 ml, intravenously) followed by a continuous infusion for 8 hours of 10 percent glucose. Blood glucose levels were thus kept between 8 and 10 μmol/g. Three to four hours after the start of the glucose administration the animals were extubated.
10. A needle 0.4 mm in diameter was inserted into the left caudate nucleus 0.5 mm anterior, 2.5 mm lateral, and 4 mm ventral to bregma (*21*). Six animals received 40 μg of AP7 (Amersham) dissolved in 2 μl of saline and neutralized with NaOH to *p*H 7.0. Six animals were injected with 2 μl of saline. The injections were performed over a period of 10 minutes. After an additional 10 minutes the needle was removed. The time from the injection to isoelectric EEG was 43 ± 10 and 38 ± 9 minutes for the AP7 and saline groups, respectively. There were no differences between the two groups in blood glucose, mean arterial blood pressure, pO_2, pCO_2, *p*H, or rectal temperature during the coma period.
11. Seven days after the hypoglycemic insult, the animals were anesthetized with halothane (3 percent) and transcardially perfused with 4 percent formaldehyde buffered to *p*H 7.4 and warmed to 37°C. The brains were removed, cut into 3-mm-thick coronal sections, processed in graded ethanol and then in xylene, embedded in paraffin, and sectioned at 8 μm (*4*). The sections were stained in a solution of celestine blue (5 g of ammonium iron sulfate, 0.5 ml of hydrochloric acid, and 5 g of celestine blue added to 100 ml of boiling distilled water), 1 percent acid fuchsine, 1 percent acetic acid.
12. The area assessed was ventral to the injection track. The acidophilic neurons were considered irreversibly damaged since they underwent cytolysis and were removed from the tissue within 6 weeks [H. Kalimo, R. N. Auer, B. K. Siesjö, *Acta Neuropathol.* **67**, 37 (1985)]. The rating of acidophilic (red) neurons and the total neuronal population was made by visual counting under the light microscope in a blind fashion. No significant shrinkage of the tissue was noted.
13. M.-L. Smith, R. N. Auer, B. K. Siesjö, *Acta Neuropathol.* **64**, 319 (1985).
14. Statistical differences among groups were calculated by using analysis of variance and a post hoc Newman-Keuls test.
15. Using a microdialysis technique monitoring the extracellular levels of amino acids in the striatum during hypoglycemia, we found that the levels of glutamate and aspartate increased ninefold and 15-fold, respectively (T. Wieloch, U. Tossman, U. Ungerstedt, unpublished results). In contrast, cerebral ischemia is associated with a significantly larger increase in glutamate than in aspartate [H. Beneveniste *et al.*, *J. Neurochem.* **43**, 1369 (1984)].
16. T. Wieloch *et al.*, *Neurosci. Lett.* **56**, 25 (1985).
17. R. Auer *et al.*, *Acta Neuropathol.*, in press.
18. J. W. Olney, *Kainic Acid as a Tool in Neurobiology* (Raven, New York, 1978), p. 95; R. Schwarcz, W. O. Whetsell, Jr., R. M. Mangano, *Science* **219**, 316 (1983).
19. B. K. Siesjö and T. Wieloch, *Br. J. Anaesth.* **57**, 47 (1985).
20. R. P. Simon, J. H. Swan, T. Griffiths, B. S. Zeldrum, *Science* **226**, 850 (1984).
21. G. Paxinos and C. Watson, *The Brain in Stereotaxic Coordinates* (Academic Press, New York, 1982).

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