Acute Axonal Dystrophy Caused by Fluorocitrate: The Role of Mitochondrial Swelling

Abstract. *Fluorocitrate, a Krebs cycle inhibitor, induces neurons to rapidly expel multitudinous lysosomes, mitochondria, and other cytoplasmic constituents into their axons. After convulsive seizures commence, spectacular axonal balloons develop owing to obstruction of axonal flow by "log jams" of extruded organelles. A swelling of neuronal mitochondria is apparently responsible for the disgorgement of cytoplasmic material into axons.*

Axonal dystrophy is a distinct neuropathological entity which is characterized by a series of swellings along the axis cylinder. In certain instances, at least, these swellings contain numerous normal and abnormal mitochondria, dense bodies, and neurofilaments (*1, 2*). Axonal dystrophy occurs in a variety of clinical and experimental affections of the nervous system (*1–3*), but its pathogenesis remains obscure. Recently it has been proposed that axonal dystrophy is due to some specific, although unknown, aberration of axonal metabolism leading to a disturbance in the proximodistal flow of axoplasm (*1*). However, the frequent coincidence of axonal dystrophy and neuronal disease suggests that axonal dystrophy is not an intrinsic axonal disorder, but a manifestation of metabolic injury to the nerve cell body.

This report describes the experimental production of axonal dystrophy with fluorocitrate (FC), and presents findings which elucidate the pathogenesis of this lesion. Fluorocitrate is a potent inhibitor of the citric acid cycle which acts by blocking the aconitase-catalyzed interconversion of citrate and isocitrate (*4*). Fluorocitrate was given to cats in a dose of 3 to 50 μg of the active isomer (dissolved in 0.003 to 0.05 ml of water) by injection into the lumbar subarachnoid space in order to restrict its action to the caudal portion of the neuraxis (*5*). After a latent period of 1 to 2 hours convulsive movements appear in the tail, hindlimbs, and trunk, culminating in a remarkable clonic-tonic seizure disorder of the (thoraco) lumbosacral spinal cord which may persist for many hours.

Lumbosacral spinal cord was fixed *in situ* by vascular perfusion with phosphate-buffered 4 percent paraformaldehyde or cacodylate-buffered 4 percent glutaraldehyde, and processed for histological, cytochemical, and ultrastructural studies (*6*). In routine paraffin sections structural changes become evident in gray matter only after convulsive seizures have been present for a time, usually 4 to 6 hours after a 10-μg dose of FC. These include blurring or fragmentation of Nissl bodies, swelling and diminished basophilia of nucleoli, clumping of neuronal and glial chromatin, and spongy state of neuropil.

Fixed-frozen sections stained for acid phosphatase activity (*6*) reveal a notable dislocation of lysosomal particles into axons long before the aforementioned structural lesions become discernible. In glutaraldehyde-fixed tissue from control cats receiving 0.01 ml of water by subarachnoidal injection, enzyme product is largely restricted to lysosomes and lipofuscin pigment granules. These are most abundant in perikarya and dendrites of neurons, but the proximal portions of axons and the perikarya of glia in the neuropil also contain reactive lysosomes. In paraformaldehyde-fixed tissue, the overall staining for acid phosphatase activity is more intense because enzyme product is diffuse as well as particulate. The diffuse reaction is an artifact produced by the potassium phosphate buffer, as it is not seen when the buffer is omitted from the fixing medium.

In FC-poisoned spinal cord, implantation cones and emergent axons of gray matter are rendered conspicuous by a localized accumulation of reactive lysosomes within 15 to 60 minutes of drug injection, and axons, both myelinated and unmyelinated, in neuropil acquire increased numbers of lysosomes. After convulsive seizures commence, axonal balloons or dilatations develop, usually 3 to 4 hours after a small dose, or 1 to 2 hours after a large dose (30 to 50 μg) of FC, and subsequently increase in size and numbers (Fig. 1). Axonal balloons usually occur in the initial segment of the axon which is without birefringent myelin when viewed in the polarizing microscope. The axonal balloons, together with the intervening axonal segments, are boldly delineated by heavy accumulations of acid phosphatase reaction product which are mainly diffuse in paraformaldehyde-fixed tissue, and both particulate and diffuse in glutaraldehyde-fixed tissue. Many myelinated axons distal to balloons (Fig. 1, C–F), as well as axons without balloons, also contain abundant enzyme product. Restricted during the early hours to the neuropil, intraaxonal reaction product extends distally to the root exit zone by 24 hours after a small dose (6 μg) of FC. Axonal balloons also are well demonstrated in preparations stained for the lysosomal hydrolases, β-glucuronidase, and acid esterase, and for the mitochondrial enzymes, nicotinamide adenine dinucleotide diaphorase, lactate dehydrogenase, and malate dehydrogenase (*6*).

The principal ultrastructural changes observed in the electron microscope during the latent period preceding convulsions are: (i) a swelling of mito-

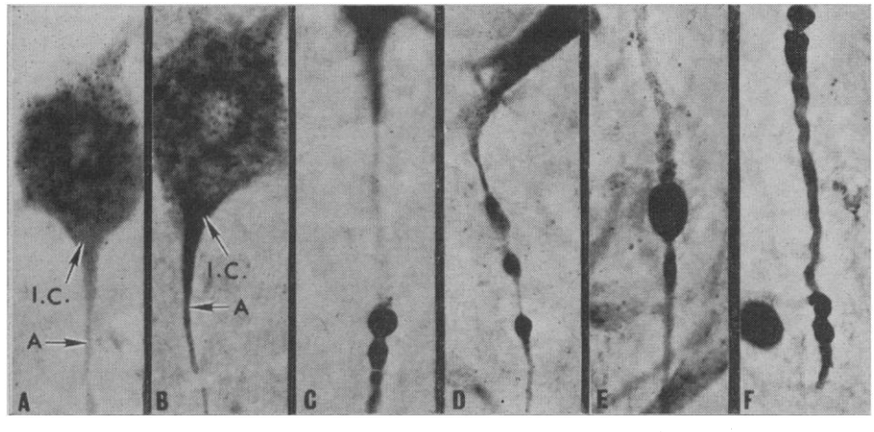

Fig. 1. Gray matter of cat spinal cord incubated in Gomori's lead glycerophosphate medium for acid phosphatase activity (*6*). Paraformaldehyde fixation (× 520). (A) Motoneuron from control cat. The implantation cone (*I.C.*) and axon (*A*) are faintly stained. (B) Motoneuron 1 hour after fluorocitrate (10 μg) injection. The implantation cone and initial segment of the axon contain an accumulation of reactive lysosomal material. (C–F) Dystrophic axons 4 hours after fluorocitrate (10 μg) injection. Acid phosphatase reaction product fills axons and axonal balloons.

chondria in the perikaryon and dendrites of neurons, and in glia (Fig. 2, A–C), and (ii) the appearance of abnormal numbers of mitochondria, dense bodies, neurofibrils, and other structures within some axons (Fig. 2D) and nerve endings. Mitochondria within axons and nerve endings are unaltered early, possibly because these structures are impermeable to FC; later some of these are swollen. After convulsions commence, the mitochondrial swelling and the axonal abnormalities become more marked and more widespread, and axonal balloons develop. Axonal balloons occur in nonmyelinated or thinly myelinated portions of axons. They are stuffed wtih various structures among which the following can be identified: mitochondria, many of which are swollen and degenerating; dense and multilamellated lysosome-like bodies; vesicles and other membranous structures; and neurofibrils (Fig. 2D). Nerve endings frequently acquire these structures also. Other fine structural lesions are: dissociation of polyribosomes in neurons and glia, swelling of endoplasmic reticulum, clumping of nuclear chromatin, loosening and disorganization of compact myelin, and astrocytic swelling.

Fluoroacetate (FA) is a potent convulsant when given systemically. However, FA is not readily converted to FC in brain tissue in vitro (*4*), and is less than one-hundredth as toxic as FC when injected intracerebrally (*7*). We found that FA, when injected into the lumbar cistern of the cat, causes epileptiform seizures and neuropathological lesions indistinguishable from FC. However, the minimum effective dose, 1.5 to 2 mg, is 300 to 400 times greater than that of FC. This seems to be a direct effect of FA on nervous tissue, as it is largely confined to that portion of the neural axis close to the injection site. It seems likely, therefore, that FA is converted to the toxic derivative, FC, to a limited extent in cat spinal cord. Fluoroacetate causes mitochondrial swelling in heart muscle, possibly because of the osmotic effect of accumulated citrate (*8*), but reportedly produces slight and infrequent cerebral lesions when given systemically to rats in convulsant doses (*9*).

The first phase of the axonal dystrophy features a convergence of lysosomes at the implantation cone and their distal migration, together with mitochondria, neurofibrils, and other cytoplasmic components, into the axon. From the exuberance of cytoplasmic organelles and the rapidity with which they accumulate in the implantation cone and the initial segment and neuropilar portion of axons, together with their later arrival in the axonal lumen at the cord periphery, we conclude that the mechanism underlying the dystrophic process is a disgorgement of cytoplasmic material into the axon, and not a "primary axostasis" or obstruction of axoplasmic flow due to increased axoplasmic viscosity or gelation, as has been postulated (*1*).

It is here proposed that the FC-induced swelling of neuronal mitochondria initiates the efflux of cytoplasmic constituents into the axon, and provides the propulsive force for their distal migration. Mitochondria collectively occupy a substantial portion of the neuronal perikaryon, perhaps 10 percent of the total volume. Therefore, a rapid expansion of the mitochondrial compartment would necessarily result in a displacement of a comparable volume of cytoplasmic material into the axon. This inference is, of course, based on the reasonable assumption that the shift of water from the neuroplasm into mitochondria is accompanied by an entry of water into the perikaryon from extraneuronal sources to maintain osmotic equilibrium.

The advent of the epileptic state initiates a second phase in the dystrophic process which is associated with a further swelling of neuronal mitochondria and an augmented expulsion of cytoplasmic material into axons. The subsequent development of axonal balloons is attributable to local "log jams" of extruded organelles blocking the axonal lumen, coupled with an enhanced transport of axoplasm distad, and the distensibility of the unmyelinated initial segment. This phase is ascribed to the seizure-associated increment in mitochondrial swelling. This swelling probably is due to a depletion of mitochondrial adenosine triphosphate engendered by the increased energy requirements of epileptic neurons whose oxidative metabolism via the Krebs cycle is already compromised. I have found that oxygen consumption and adenosine triphosphate concentration in FC-poisoned brains of rats are normal during the latent period, but decline sharply after seizure activity appears (*10*). Interference with the respiration-coupled generation of adenosine triphosphate by various means is known to result in mitochondrial swelling (*11*).

Other factors may conceivably contribute to the dystrophic process in FC-poisoning, such as: (i) chemical injury to the axolemma and axonal contents resulting from the action of lysosomal enzymes; (ii) increased microperistaltic activity of neurons; (iii) segmental contraction of the axolemmal membrane in response to stretch; (iv) increased electrophoretic convection; and (v) astroglial swelling. However, the significance of these factors cannot presently be assessed.

A swelling of neuronal mitochondria may be of general importance as a

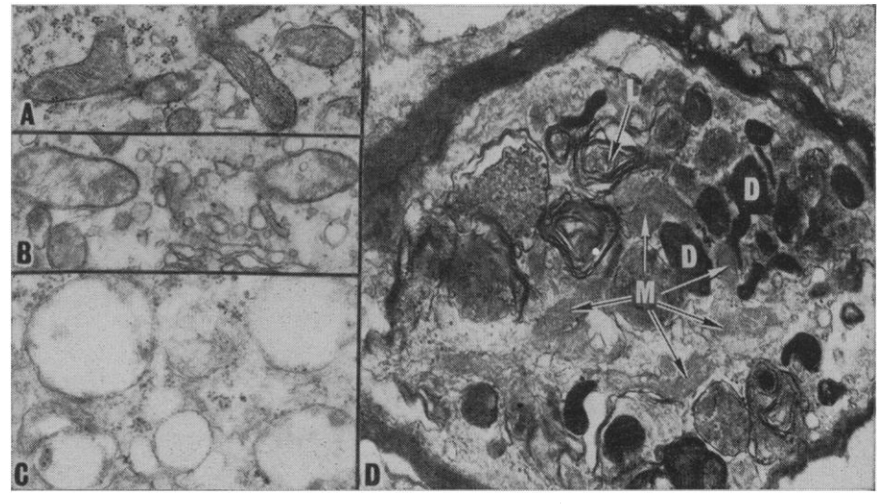

Fig. 2. Electron micrographs of spinal gray matter after fluorocitrate (10 μg) injection. (A–C) Neuronal cytoplasm showing mitochondria (× 21,000). (A) Control. (B) One hour after FC. Mitochondria are moderately swollen. (C) Four hours after FC. Severely swollen mitochondria are sac-like structures; the double membrane is still evident, but the cristae are obliterated, and the matrix is electronlucent. (D) Myelinated axon 4 hours after FC (× 15,300). The axon is filled with dense (*D*) and multilamellated (*L*) lysosomal bodies, degenerating mitochondria (*M*), vesicles, neurofibrils, and unidentifiable material.

mechanism for the acute production of axonal dystrophy. Mitochondrial swelling has been noted together with axonal dystrophy following neuronal injury by x-irradiation (Andres, see *2*; Forssmann *et al.*, *12*) and by plasmocid (D'Agostino, see *2*) and cyanide (*13*, *14*) poisoning. However, these two lesions have not hitherto been causally linked. I have found that several metabolic inhibitors with different modes of action, including cyanide, arsenious oxide, ouabain, and methionine sulfoximine, when injected into the lumbar theca of cat, cause mitochondrial swelling in spinal neurons and dystrophic changes in their axons (*14*).

Although the proximodistal transport of axonal material is a well-documented physiological phenomenon, the mechanism responsible for this transport is still obscure (*15*). The present study may shed some light on this subject. It is known that the respiratory enzymes embedded within the mitochondrial membrane, in addition to catalyzing the coupled generation of adenosine triphosphate, perform osmotic and mechanical work involving conformational changes in the mitochondrion (*11*). Respiration-linked mechanochemical changes in mitochondria analogous to the "small amplitude" swelling-contraction cycles produced in vitro (*16*) and the configurational and volumetric changes observed in fibroblast mitochondria in tissue culture (*17*) probably occur in nervous tissue. Thus it seems reasonable to envisage neuronal (and axonal) mitochondria as pulsating organelles with a pump-like action that serves to propel the axonal stream in a proximodistal direction.

HAROLD KOENIG

Veterans Administration Research Hospital and *Department of Neurology and Psychiatry, Northwestern University Medical School, Chicago, Illinois 60611*

References and Notes

1. S.-M. Chou and H. A. Hartmann, *Acta Neuropathol.* **3**, 428 (1964); O. W. Sacks and W. J. Brown, *Bull. Los Angeles Neurol. Soc.* **31**, 35 (1966).
2. A. N. D'Agostino, *Neurology* **14**, 114 (1967); K. H. Andres, *Z. Zellforsch.* **61**, 1 (1963); G. Ule, *ibid.* **56**, 130 (1962); P. Lampert and A. Pentschew, *Acta Neuropathol.* **4**, 158 (1964); R. D. Terry, N. K. Gonatas, M. Weiss, *Amer. J. Pathol.* **44**, 269 (1964).
3. G. Wohlfahrt, *Lunds Univ. Arsskr., Avd. 2*, **56**, 3 (1959); F. Seitelberger, *3rd International Congress of Neuropathology at Brussels* (Edition Acta Medica Belgica, Brussels, 1957), p. 127; J. H. Sung, *J. Neuropathol. Exp. Neurol.* **23**, 567 (1964); ——— and E. M. Stadlan, *ibid.* **25**, 341 (1966); R. L. Friede, *Acta Neuropathol.* **4**, 288 (1965); I. Klatzo, D. C. Gadjusek, V. Zigas, *Lab. Invest.* **8**, 799 (1959); D. Cowen, and E. V. Olmstead, *J. Neuropathol. Exp. Neurol.* **22**, 175 (1963); G. Ule, in Henke-Lubarsch, *Handbuch der Speziellen Pathologischen Anatomie und Histologie* (Springer, Berlin, 1956), vol. 13, sect. 1A, p. 950.
4. R. A. Peters, *Advan. Enzymol.* **18**, 113 (1957); ———, *Biochemical Lesions and Lethal Synthesis*, P. Alexander and Z. M. Bacq Eds. (Pergamon, Oxford, 1963), p. 88.
5. A crude synthetic preparation of DL-fluorocitrate containing about 11 percent of the active isomer and kindly supplied by Sir R. A. Peters was used for some of these experiments. The barium salt of DL-FC supplied by Sigma Chemical Co. was also used after removal of the barium by precipitation as the sulfate, with identical results. The drug was injected intrathecally after an L-7 laminectomy performed under pentobarbital anesthesia. The animal was kept in the head-up position to reduce rostral flow of FC.
6. The 4 percent paraformaldehyde fixative contained 7 percent sucrose and .015*M* potassium phosphate buffer, *p*H 7.4. The 4 percent glutaraldehyde solution contained 0.1*M* cacodylate buffer, *p*H 7.4. Perfuse-fixed spinal cord was placed in paraformaldehyde for 24 hours, or glutaraldehyde for 4 hours, at 4°C, rinsed, and stored in cacodylate-buffered 7 percent sucrose [D. Sabatini *et al.*, *J. Cell Biol.* **17**, 19 (1963)] or in a gum-sucrose medium [S. Holt, *Exp. Cell Res. Suppl. No. 7*, 1 (1959)] at 4°C until used. Paraffin sections were stained by standard Nissl and myelin methods. Aldehyde-fixed frozen sections were stained for the following enzyme activities: acid phosphatase [G. Gomori, *Microscopic Histochemistry* (Univ. of Chicago Press, Chicago, 1952); β-glucuronidase [M. Hayashi *et al.*, *J. Histochem. Cytochem.* **12**, 293 (1964)]; acid esterase in the presence of .0001*M* diisopropyl-phosphofluoridate [A. Pearse, *Histochemistry* (Little, Brown, Boston, ed. 2 1960)]; NAD diaphorase, lactate and malate dehydrogenases with nitro B tetrazolium (A. Pearse, *ibid.*). Tissues incubated for these activities in the absence of the specific substrates showed no reaction. For electron microscopy, tissues were post-fixed in 1 percent buffered OsO_4 at 4°C and embedded in araldite, and ultrathin sections were stained with uranyl acetate and/or lead citrate, and examined in an RCA EMU3F electron microscope. In a few experiments nonfrozen sections [R. Smith and M. Farquhar, *Nature* **200**, 691 (1963)] of paraformaldehyde-fixed tissue were incubated in Gomori's medium for acid phosphatase activity, and then processed for electron microscopy.
7. P. L. Morselli, S. Garattini, F. Marcucci, E. Mussini, W. Rewersky, L. Valzelli, R. A. Peters, *Biochem. Pharmacol.* **17**, 195 (1968).
8. I. Pucci, *Exp. Cell Res.* **35**, 412 (1964); R. Bovis, F. H. Kasten, T. Okigaki, *ibid.* **43**, 611 (1966).
9. S. P. Hicks, *Arch. Pathol.* **49**, 111 (1950).
10. H. Koenig and A. Patel, *J. Cell Biol.* **35**, 72A (1967); A. Patel and H. Koenig, in preparation.
11. Reviewed in A. L. Lehninger, in *Horizons in Biochemistry*, M. Kasha and B. Pullman, Eds. (Academic Press, New York, 1962), p. 421.
12. W. G. Forssmann, H. Tinguely, J. M. Posternak, C. Rouiller, *Z. Zellforsch.* **72**, 325 (1966).
13. A. Hirano, S. Levine, H. M. Zimmerman, *J. Neuropathol. Exp. Neurol.* **26**, 200 (1967); H. Hager, W. Hirschberger, W. Scholz, *Aerospace Med.* **31**, 379 (1960).
14. H. Koenig, *J. Neuropathol. Exp. Neurol.* **28**, 173 (1969); and unpublished observations.
15. Reviewed in S. Ochs, in *Macromolecules and Behavior*, J. Gaito, Ed. (Appleton-Century-Crofts, New York, 1966), p. 20.
16. F. A. Holton, *Biochem. J.* **66**, 37P (1957); L. Packer *J. Biol. Chem.* **235**, 242 (1960).
17. J. Frederic, *Arch. Biol.* (*Liege*) **69**, 167 (1958).
18. These studies were supported by the Veterans Administration, and by grants from the National Multiple Sclerosis Society (MS-512-B-7) and NIH (NB05509, NB06838 and NB01456). I acknowledge the valuable contributions of Mrs. B. S. Newson and Mr. C. T. Hughes to the electron microscopic aspects of this study. I am also indebted to Misses G. T. Ronga, S. M. Koenig, and L. E. Koenig, and Mrs. D. L. Thomas for the light microscopic preparations, and to Mr. E. Washington for aid in carrying out the animal experiments.

4 December 1968; revised 24 January 1969

Complementation Analysis on Virus-Fused Chinese Hamster Cells with Nutritional Markers

Abstract. *Cell fusion experiments have been carried out with Chinese hamster cell mutants with different nutritional growth requirements. Conditions have been devised in which approximately 1 to 2 percent of the cell population remaining after fusion are fused, hybrid cells. The all-or-none nature of the genetic markers employed and the extremely low reversion rates insure that no contamination of the hybrid population with parental forms occurs. Hybrids between glycine- and hypoxanthine-requiring mutants are prototrophic, which indicates that both mutations are recessive. Hybrids between a glycine-deficient mutant and a single-step mutant which requires glycine, hypoxanthine, and thymidine are relieved of the glycine dependency, an indication that the two loci associated with glycine dependence are different. This mutation to the triple-supplement requirement as well as a proline deficiency were also shown to be recessive mutations. The system appears applicable to a variety of genetic problems.*

Experiments have been described (*1*) in which single-step gene mutations could be induced in Chinese hamster cells grown in tissue culture for long periods and possessed of reasonably stable karyotypes. These mutations are produced by standard mutagens such as ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; they have spontaneous reversion frequencies varying between 10^{-6} and 2×10^{-8}; their corresponding reverse mutations can also be elicited by standard mutagens; and the nutritional requirements introduced appear to be absolute, since no growth whatever is exhibited by deficient mutants in the absence of the specific supplement. In the case of a spontaneous proline-deficient mutation, it was demonstrated that the mutant makes no proline, that the block in the biosynthetic chain lies in the step converting glutamic acid to its gamma-semialdehyde, and that the revertant to proline independence obtained from the mutant cell behaves like a hetero- or a hemizygote with respect to this gene (*2*). Availability of these markers makes possible application of the cell fusion technique (*3*) to obtain rapid and convenient quantitation and isolation of hybrid cells carrying combined genetic markers, without contamination