Different Proteins Associated with 10-Nanometer Filaments in Cultured Chick Neurons and Nonneuronal Cells

Abstract. *A protein of molecular size 180 kilodaltons is associated with 10-nanometer filaments in neurons and is immunologically distinct from smaller putative neurofilament subunits and from 10-nanometer filament proteins in nonneuronal cells, such as myotubes and fibroblasts. Neurons do not contain vimentin, the major filament protein in many other cells, including the nonneuronal cells in cultures of neural tissue.*

The relationship of the protein subunits of 10-nm neurofilaments (NF's) to 10-nm intermediate filament (IF) subunits in other cells has not been fully elucidated. Electrophoretic analysis of the polypeptides found in NF-rich fractions suggests that NF's of the mammalian central nervous system (CNS) and peripheral nervous system (PNS) consist of three polypeptides of molecular size 68, 150, and 200 kilodaltons (kd) (*1–4*). Neurofilament preparations from the CNS contain, in addition, components of molecular size 58 and 51 kd. The 51-kd protein is absent from NF preparations from the PNS and has been ascribed to astrocyte IF subunits (*3*), also termed glial fibrillary acidic protein (GFA) (*5*). However, there may also be breakdown products of the NF triplet around 50 kd (*3, 6*). In contrast, IF subunits in most nonneuronal cells are in the range 50 to 60 kd (*7–11*). Furthermore, fibroblasts and skeletal muscle have been reported to contain, in addition to other IF proteins, the 68-kd NF polypeptide (*12*).

Some antiserums to various brain filament preparations contain antibodies that bind only to neurons (*3, 9, 13–15*), an indication that at least some NF proteins are not related to IF proteins of other cell types. With few exceptions, the identity of the polypeptides reacting with a given antiserum is unknown. One antiserum to NF reacted with a 68-kd molecule, but binding to other subunits was not ruled out (*13*). In another study, antiserums to individual members of the putative NF triplet (*3*) each bound to all three NF polypeptides as well as to a 51-kd protein; this result suggests that the three NF proteins share antigenic determinants and may be derived from one another.

Antigenic similarity between NF's and the IF's of different cell types has also been reported. Some antiserums to putative NF proteins bind to endothelial cells (*16*), cardiac myocytes (*16*), astrocytes (*17*), fibroblasts (*12*), and skeletal muscle (*12*); however, the antiserums used in two of these studies were later judged not to be directed against NF's (*3*). Furthermore, antiserums to certain 50- to 60-kd subunits of other cells also bind to neurons (*18*).

Several questions about NF subunits remain, including whether the three putative NF subunits are actually components of NF; whether these three polypeptides are structurally related; and whether NF's contain proteins common to other IF's. We now present evidence that in the chicken (i) a 180-kd protein is associated with NF and is antigenically distinct from two other putative NF subunits of 160 and 70 kd; (ii) NF's do not contain any of four known distinct IF subunits found in various nonneuronal cells [vimentin (*9, 11*), desmin (*8, 10*), GFA (*5, 14*), and cytokeratin (*11*)]; (iii) the nonneuronal (glial) cells in cultures of the chick nervous system do not contain a protein similar to the 180-kd NF subunit, but do contain vimentin, as do fibroblasts, chondroblasts, presumptive myoblasts, immature myotubes, and several other cell types (*19*).

Rabbits were immunized with a preparation of adult chicken brain filaments (*9*). Their serums were screened for the presence of antibodies to neuronal filaments by indirect immunofluorescence on cultures of different types of dissociated embryonic chick cells (*20*).

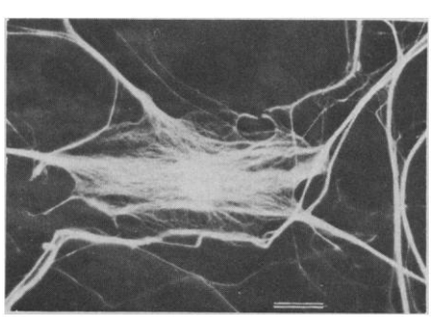


Fig. 1. Fluorescence micrograph of a chick spinal cord culture stained with antiserum to NF. Spinal cord from 6-day embryos was dissociated and grown on collagen-coated Aclar (Allied Chemical Co.) squares, in 35-mm tissue culture dishes for 3 weeks. The culture was fixed in 2 percent formaldehyde, made permeable with 0.5 percent Triton X-100 in phosphate-buffered saline (PBS-TX) and incubated in antiserum to NF (1:250) and then in rhodamine-conjugated goat antibody to rabbit immunoglobulin G (IgG) (1:250). The PBS-TX was used for dilutions and washings. Antibody is bound only to neuronal perikarya and processes. The confluent nonneuronal cells that lie beneath the neuron are unstained and barely visible. Within the neuronal perikaryon, antibody is localized on filamentous structures that extend into processes. Scale bar, 20 μm.

Antiserum to NF from one rabbit, which bound to filaments in neurons when it was tested on cultures of spinal cord, did not bind to any nonneuronal (glial) cells as judged by staining (Fig. 1). This antiserum did not bind to cultured fibroblasts, presumptive myoblasts, skeletal, cardiac, or smooth muscle, chondrocytes, or hepatocytes (not shown). Identical results were obtained with sections from 18-day embryos (*21*).

The following observations rule out the possibility that the antiserum to NF contained antibodies to microtubules or actin microfilaments: the antiserum did not bind to the microtubules of the spindle apparatus in mitotic cells or to stress fibers in any cell type tested; and the filaments stained by the antiserum remained intact after treatment of spinal cord cultures with cytochalasin B, which disrupts microfilaments, and with Colcemid, which disrupts microtubules. Finally, electron microscopy of an immunohistochemical preparation labeled with peroxidase confirmed the binding of these antibodies to 10-nm filaments in neurons (Fig. 2D).

For further characterization of antiserum to NF, we prepared NF fractions from CNS and PNS tissues (*22*). These fractions contained major components at 70, 160, and 180 kd (Fig. 3A), values which differ slightly from those found for the NF triplet of mammalian polypeptides. Absorption of antiserum to NF with the NF preparations from either the PNS or CNS abolished its capacity to bind to neurons in immunofluorescence, indicating that the NF preparations contained all the antigens against which the immunohistochemically detectable antibodies in this serum were directed.

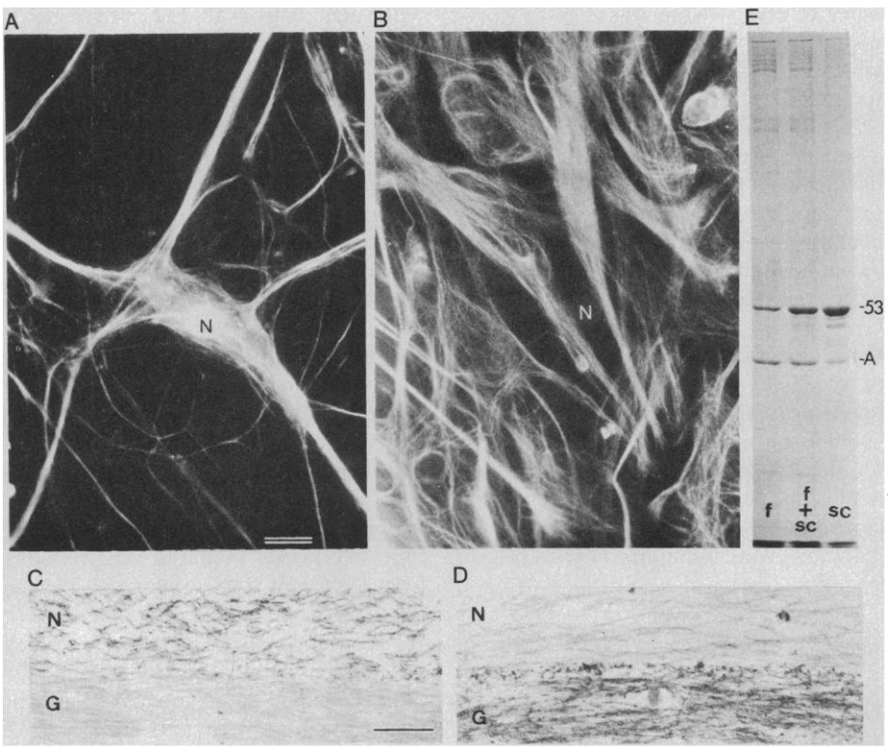
To identify the specific components in the NF fractions that would bind antibodies, we carried out immunolabeling of the proteins after electrophoretic separation in agarose-acrylamide gels (Fig. 3B). After incubation of the gel with antiserum to NF and ^{125}I-labeled staphylococcal protein A, only one labeled band was obtained—at 180 kd. When solubilized total spinal cord proteins were applied to the gel, instead of NF-rich fractions, no additional labeled bands were detected. These results demonstrate the molecular specificity of the antiserum to NF as well as antigenic differences between the 180-kd polypep-

0036-8075/81/0501-0567$00.50/0

tide and other putative NF subunits. The immunohistochemical results thus (i) provide direct evidence for the association of the 180-kd protein with NF's and (ii) show that IF's of nonneuronal cells do not contain this NF subunit.

The presence of unique antigenic determinants on the 180-kd subunit suggests that the 70- and 160-kd subunits are not derived from the 180-kd protein. Although our results do not exclude the possibility that smaller polypeptides are generated from the 180-kd molecule in such a way that the antigenic site recognized by this antiserum to NF is altered, our interpretation is supported by the demonstration (*23*) of unique antigenic sites on the 70-kd molecules as well (*24*).

To determine whether any of the IF subunits present in other cell types are also present in neurons, we used antiserums specific for different IF subunits. The major fibroblast IF subunit (vimentin) is found in chondrocytes, pigment cells, replicating presumptive myoblasts, and immature skeletal myotubes (*9, 19, 20*). When 3-week spinal cord cultures were examined with antiserum to vimentin (*25*), neuronal structures were unstained, whereas IF's in virtually all glial cells were visualized. Similarly, in sections of spinal cord from 18-day embryos, antibodies to vimentin bound only to glial cells. That the binding of antibodies to vimentin was restricted to nonneuronal cells is shown by double-staining with antibodies to vimentin and antiserum to NF (Fig. 2, A and B). Electron microscopy shows that the antiserum to NF binds to the 10-nm IF only in neurons and that antibodies to vimentin bind to the 10-nm IF only in glia (Fig. 2, C and D). These results demonstrate that there is no vimentin in neurons and that antibodies to vimentin do not react with NF



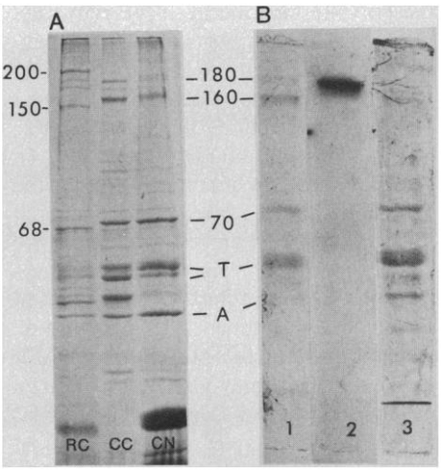


Fig. 2. (A and B) Fluorescence micrographs of a single field in a spinal cord culture similar to that in Fig. 1, double-stained with (A) antibody to NF and (B) antibody to vimentin. Fixation and incubation were as in Fig. 1, except that the first incubation was in rabbit IgG antibody to NF plus mouse IgM antibody to vimentin (*25*), and the second incubation was in rhodamine-conjugated goat antibody to rabbit IgG plus fluorescein-conjugated goat antibody to mouse IgM. The neurons and processes revealed by antibody to NF [rhodamine-selective filters in (A)] are not stained by antibody to vimentin [fluorescein-selective filters in (B)]; conversely, antibody to NF does not bind to the nonneuronal cells, but antibody to vimentin does (*N* is the neuronal nucleus). Scale bar, 20 μm. (C and D) Electron micrographs showing (C) binding to IF of antibody to NF in neuronal processes, but not in glial processes and (D) binding to IF of antibody to vimentin in glial processes, but not in neuronal processes. Spinal cord cultures similar to those in Fig. 1 and in (A) and (B), were incubated in (C) antibody to NF or (D) antibody to vimentin and then in peroxidase-antiperoxidase (Cappel). After the preparation was developed in diaminobenzidine, light microscopy confirmed that binding of antibody to NF was restricted to neurons and binding of antibody to vimentin was restricted to glia. Cultures were then postfixed in glutaraldehyde and OsO_4, embedded, and sectioned for electron microscopy. The fields shown contain a portion of a neurite (*N*) flanked by a glial process (*G*). (Membrane boundaries between the cells were not preserved by the mild fixation and detergent treatment prior to incubation with antibodies.) Glial processes contain characteristic dense bundles of IF, which are covered with heavy reaction product after incubation with antibody to vimentin (D), but are unlabeled after incubation with antibody to NF (C). Neurites contain sparser IF, which are coated with reaction product when incubated with antibody to NF (C), but remain unlabeled when incubated with antibody to vimentin (D). Scale bar, 1 μm. (E) Electrophoretic separation of proteins in Triton-insoluble cytoskeletons of a chick fibroblast culture (*f*) and a spinal cord culture similar to those in Fig. 1 and (A) to (D) (*SC*). A mixture of the two (*f* + *SC*) shows that the mobility of the major band in the spinal cord culture is identical to that of chick fibroblast vimentin (53 kd); *A*, actin. Fig. 3. (A) Electrophoretic separation of proteins in NF-rich fractions from rat spinal cord (*RC*), chicken spinal cord (*CC*), and chicken sciatic nerve (*CN*) (*22*). The rat NF preparation (*RC*) contains the major bands at 68, 150, and 200 kd that have been designated NF triplet polypeptides (*1–4, 13*). Chicken NF preparations (*CC* and *CN*) contain, instead, major bands at 70, 160, and 180 kd. The positions of actin (*A*) and of α- and β-tubulin (*T*) were established with pure actin and tubulin standards in another lane. The band at 180 kd is single in some chicken NF preparations (for example, *CC*) and a doublet in others (for example, *CN*); both forms have been obtained with spinal cord and sciatic nerve. Electrophoresis was in 10 percent acrylamide, 0.26 percent bisacrylamide, and 0.1 percent SDS gels, with the buffer system of Laemmli (*26*). (B) Labeling with antiserum to NF of the electrophoretically separated chicken spinal cord NF preparation (*CC*) shown in (A). The buffer composition was as in (A), but gels consisted of 0.8 percent agarose and 8 percent acrylamide, cross-linked with *N,N'*-diallyltartardiamide. After electrophoresis and fixation in 25 percent isopropanol and 10 percent acetic acid, the acrylamide was depolymerized with 2 percent periodic acid (*27*). One slice of the resulting agarose gel was then stained with Coomassie blue (lane 1). A duplicate slice (lane 3) was incubated in antiserum to NF (1:10), followed by ^{125}I-labeled staphylococcal protein A (1 μCi/ml per gel slice; Amersham), stained, dried, and exposed to Kodak X-Omat R film (*28*). The developed autoradiograph (lane 2) shows binding of antibody only to a band at 180 kd. When the NF preparation contained a doublet at the 180-kd position, both bands were labeled (not shown). Incubation in serum obtained before rabbits were immunized (1:10) did not produce labeled bands.

subunits. Similarly, neurons did not bind antibodies to muscle-specific (desmin), epithelium-specific (cytokeratin), or astrocyte-specific (GFA) IF subunits (data not shown). The binding of antibodies to vimentin to the nonneuronal cells in spinal cord cultures suggests that these cells contain a protein similar to vimentin. Additional evidence for this was obtained by analyzing the detergent-insoluble residue of such cultures on sodium dodecyl sulfate gels (Fig. 2E). The major band was one that migrated with chick vimentin.

GUDRUN S. BENNETT
STEPHEN J. TAPSCOTT
FREDRIC A. KLEINBART
PARKER B. ANTIN
HOWARD HOLTZER
Department of Anatomy, University of Pennsylvania Medical School, Philadelphia 19104

References and Notes

1. P. N. Hoffman and R. J. Lasek, *J. Cell Biol.* **66**, 351 (1975).
2. W. W. Schlaepfer and L. A. Freeman, *ibid.* **78**, 653 (1978).
3. R. K. H. Liem, S.-H. Yen, G. D. Salomon, M. L. Shelanski, *ibid.* **79**, 637 (1978).
4. M. S. Runge, H. W. Detrich III, R. C. Williams, Jr., *Biochemistry* **18**, 1689 (1979).
5. L. F. Eng, J. J. Vanderhaegen, A. Bignami, B. Gerstl, *Brain Res.* **28**, 351 (1971).
6. W. W. Schlaepfer and S. Micko, *J. Cell Biol.* **78**, 369 (1978); W. A. Day, *J. Ultrastruct. Res.* **70**, 1 (1980).
7. H. Holtzer, S. Fellini, N. Rubinstein, J. Chi, K. Strahs, in *Cell Motility*, R. Goldman, T. Pollard, J. Rosenbaum, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976), p. 823; J. V. Small and A. Sobieszek, *J. Cell Sci.* **23**, 243 (1977); S. Brown, W. Levinson, J. A. Spudich, *J. Supramol. Struct.* **5**, 119 (1976); R. O. Hynes and A. T. Destree, *Cell* **13**, 151 (1978); J. Starger and R. D. Goldman, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2422 (1977); A. Eriksson and L.-E. Thornell, *J. Cell Biol.* **80**, 231 (1979); P. Cooke, *idid.* **68**, 539 (1976).
8. E. Lazarides and B. D. Hubbard, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4344 (1976).
9. G. S. Bennett, S. A. Fellini, J. M. Croop, J. J. Otto, J. Bryan, H. Holtzer, *ibid.* **75**, 4364 (1978).
10. S. A. Fellini, G. S. Bennett, Y. Toyama, H. Holtzer, *Differentiation* **12**, 59 (1978); S. A. Fellini, G. S. Bennett, H. Holtzer, *Am J. Anat.* **153**, 451 (1978).
11. W. W. Franke, E. Schmid, M. Osborn, K. Weber, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5034 (1978); *J. Cell Biol.* **81**, 570 (1979).
12. C. Wang, D. J. Asai, E. Lazarides, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1541 (1980).
13. W. W. Schlaepfer, *J. Cell Biol.* **74**, 226 (1977); ——— and R. G. Lynch, *ibid.*, p. 241.
14. D. Dahl and A. Bignami, *J. Comp. Neurol.* **176**, 645 (1977).
15. M. Schachner, C. Smith, G. Schoonmaker, *Dev. Neurosci.* **1**, 1 (1978).
16. S. H. Blose, M. L. Shelanski, S. Chacko, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 662 (1977).
17. S.-H. Yen, D. Dahl, M. Schachner, M. L. Shelanski, *ibid.* **73**, 529 (1976).
18. A. Eriksson, L. E. Thornell, E. Lundgren, T. Stigbrand, *FEBS Lett.* **102**, 177 (1979).
19. H. Holtzer, G. S. Bennett, S. J. Tapscott, J. M. Croop, A. Dlugosz, Y. Toyama, in *International Cell Biology, 1980–1981*, H. G. Schweiger, Ed. (Springer Verlag, Berlin, in press).
20. G. S. Bennett, S. A. Fellini, H. Holtzer, *Differentiation* **12**, 71 (1978).
21. E. Schmid, S. Tapscott, G. S. Bennett, J. Croop, S. A. Fellini, H. Holtzer, W. W. Franke, *ibid.* **15**, 27 (1979).
22. Our procedure was based on those described in (*2*, *4*). Cervical spinal cord, after being freed of meninges, was homogenized either in buffer A (100 m*M* sodium phosphate, 3 m*M* Na_2-EDTA, and 2 m*M* dithiothreitol, *p*H 7.2) or in buffer B (10 m*M* tris, 3 m*M* Na_2-EDTA, and 2 m*M* dithiothreitol, *p*H 6.8). The supernatant obtained after centrifugation at 25,000*g* for 30 minutes was centrifuged at 270,000*g* for 60 minutes. The resulting pellet was washed twice and suspended in buffer A. This suspension, when examined by electron microscopy after negative staining, contained 10-nm filaments and membranous debris. Peripheral nerve filaments were obtained by osmotic shock of desheathed, minced sciatic nerve in buffer B, followed by centrifugation as above. The pellet was washed and suspended in buffer A.
23. G. S. Bennett, S. J. Tapscott, H. Holtzer, in *Changing Concepts of the Nervous System*, A. R. Morrison and P. L. Strick, Eds. (Academic Press, New York, in press).
24. The report by H. Czosnek, D. Soifer, and H. M. Wisniewski [*J. Cell Biol.* **85**, 726 (1980)], which appeared after submission of this report, provides different evidence that the three NF proteins are not synthesized as larger precursors.
25. Production and characterization of rabbit antiserum to chick vimentin (previously termed the 58-kd protein) is described in (*9*, *10*, *20*). More recent estimates of the size of chick vimentin give a lower value of 53 kd (*19*). We also used a monoclonal mouse antibody to chick vimentin (S. J. Tapscott, P. Antin, G. S. Bennett, R. H. Kennett, H. Holtzer, in preparation).
26. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
27. J. Renart, J. Reiser, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3116 (1979).
28. W. S. Adair, D. Jurivich, U. W. Goodenough, *J. Cell Biol.* **79**, 281 (1979).
29. Supported by NIH grants HL18708, HL15835, HD07152, and CA 18194 and by the Muscular Dystrophy Association. S. J. T. is the recipient of a Medical Science Insurance Scholarship from the Nationwide Insurance Co.

21 November 1980

Drug Discrimination Learning in Lead-Exposed Rats

Abstract. *Lead acetate (0.02 or 0.5 percent) was administered to dams throughout the lactation period with half of the litters continuing on lead after weaning. Drug thresholds for* d*-amphetamine were determined by using the drug-discrimination learning paradigm. All the offspring that had been exposed to lead were less sensitive to the stimulus properties of* d*-amphetamine irrespective of whether or not they had continued on lead after weaning.*

Studies of the neurobehavioral effects of long-term exposure to lead show consistently that lead-exposed rat pups exhibit altered drug responsivity. Responsivity to both agonists and antagonists of putative neurotransmitters has been examined, but most studies have been concerned with the effects of *d*-amphetamine sulfate. The altered response to an injection of *d*-amphetamine may be expressed as an attenuated increase (*1*) or paradoxical decrease (*2*) in locomotor activity relative to uninjected controls. Lead-exposed offspring have also shown altered drug responsiveness on active avoidance tasks, spontaneous alternation, and drug-elicited aggression (*3*). However, the direction of the drug response relative to controls has varied and appears to depend on such factors as the nature of the task, the dose, and the duration of exposure. Conflicting theories of a hyposensitive or hypersensitive catecholaminergic system have therefore been postulated [see (*4*)].

To address this question we used a drug-stimulus discrimination paradigm that permits assessment of drug-response thresholds (*5*). The task presented to the animal is analogous to a simultaneous discrimination problem with the only cue for directing response selection being the endogenous drug state, that is, whether the animal has received a drug or "no-drug" (vehicle) injection. We hypothesized that if a lead-exposed animal had a decreased sensitivity to amphetamine then it would have an elevated drug-response threshold relative to the controls. In contrast, increased drug sensitivity would shift the drug discriminability threshold to below that of the controls.

Twelve female Long-Evans hooded rats (Charles River; four per group) were mated at 90 days of age. Within 12 hours after parturition and for the duration of lactation they were exposed to 0, 0.02, or 0.5 percent lead acetate in their drinking water. Litters were culled to ten pups at birth and eight pups at day 3. At weaning (day 21), two males were randomly selected from each of the litters from lead-exposed dams, one male pup being exposed to the lead treatment of its dam, the other to distilled water. In addition, one male pup was randomly selected from each of the four control litters and exposed to distilled water. Remaining pups in the litters were assigned to other studies. All offspring were individually housed and maintained on Purina Lab Chow 5001. We thus had five treatment groups (four rats per group) for subsequent experimentation—namely, group 0/0, the controls; groups 0.02/0.02, offspring exposed to 0.02 percent lead acetate during lactation via the dam and after weaning; group 0.02/0, offspring exposed to lead during lactation only; and group 0.5/0.5 and group 0.5/0, offspring exposed to 0.5 percent lead acetate during the same periods as their 0.02 counterparts.

The litters exposed to 0.5 percent lead acetate had significantly depressed weights at weaning (42.41 ± 4.6, mean ± standard deviation) compared to the controls (48.6 ± 3.9) and litters exposed to 0.02 percent lead acetate (49 ± 4.4).

0036-8075/81/0501-0569$00.50/0