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10. SFFF conditions: equipment and technique as in (*2*); rotor speed, 15,000 rev/min; relaxation time, 1 minute; time delay–decay constant, 10.0 minutes; flow rate, 0.5 ml/min; mobile phase was 0.1*M* tris and 0.2*M* NaCl at *p*H 7.6.
11. For a complete discussion of time delay–exponential force field SFFF, see W. W. Yau and J. J. Kirkland [*Sep. Sci. Technol.* **16**, 577 (1981)].
12. We thank J. W. Gray, Y.-C. Tse Dinh, B. Mazur, C.-F. Chui, and G. Cordova for technical advice and discussions; J. E. Gray for laboratory protocols and facilities required for nucleic acid analysis; C. Lewis for sample preparation and gel electrophoresis; and C. H. Dilks, Jr., for assistance with the SFFF instrumentation.

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Infection-Specific Particle from the Unconventional Slow Virus Diseases

Abstract. *Scrapie-associated fibrils, first observed in brains of scrapie-infected mice, were also observed in scrapie-infected hamsters and monkeys, in humans with Creutzfeldt-Jakob disease, and in kuru-infected monkeys. These fibrils were not found in a comprehensive series of control brains from humans and animals affected with central nervous system disorders resulting in histopathologies, ultrastructural features, or disease symptoms similar to those of scrapie, kuru, and Creutzfeldt-Jakob disease. These fibrils are also found in preclinical scrapie and in the spleens of scrapie-infected mice; they are a specific marker for the "unconventional" slow virus diseases, and may be the etiological agent.*

Scrapie in sheep and goats and Creutzfeldt-Jakob disease (CJD) and kuru in humans cause fatal infectious encephalopathies after long periods of inapparent disease. The causal "slow viral" agents have yet to be identified by electron microscopy. Scrapie-associated fibrils (SAF) are distinct particulate structures first observed in scrapie-infected mouse brain preparations by negative stain electron microscopy (*1*). They are composed of two to four twisted filaments, each filament 4 to 6 nm in diameter and of variable length (*1*). Merz *et al.* have reported these structures in brain extracts from three strains of mice infected with any of six different strains of scrapie (139A, ME7, 22A, 87V, 22L, and 79A); from hamsters infected with the 263K strain of scrapie; from mice, guinea pigs, and hamsters infected with a single isolate of CJD; and from a human case of CJD and a human case of Gerstmann-Straussler syndrome (*2*), thought to be a variant of CJD (*3*).

There has so far been a good correlation between the presence of SAF and the "unconventional" slow virus diseases. We have now extended this correlation by the identification of SAF in experimental kuru and in additional cases of human CJD and scrapie. We have established the specificity of SAF by their absence in a comprehensive assortment of other human and animal diseases exhibiting either similar histopathologies, ultrastructural features, or clinical courses similar to those of scrapie, kuru, or CJD. In so doing we have also demonstrated the capability of negative stain electron microscopy to distinguish between SAF and similar structures observed in other disorders of the central nervous system (CNS).

The principal neuropathological features of Alzheimer's disease and senile dementia of the Alzheimer type (AD-SDAT) are neuritic amyloid plaques and neurofibrillary tangles composed of paired helical filaments (PHF). Neurofibrillary tangles are also associated with Guam parkinsonism dementia complex and amyotrophic lateral sclerosis (ALS) on Guam and occasional cases of ALS elsewhere. The ability to distinguish SAF from amyloid fibrils and PHF observed together or separately in these different human CNS diseases, and to corroborate cases of mixed diagnosis on the basis of these ultrastructural features, may make it possible to use ultrastructural analysis to distinguish types of CNS involvement in this complex disease spectrum.

Specimens coded with respect to species, treatment, and diagnosis from 44 separate brains were examined in three separate experiments (two at The National Institutes of Health and one at the Institute for Basic Research) with the code broken after each experiment by a third individual after all the results were tabulated. Crude mitochondrial synaptosomal preparations were prepared from 0.5 to 3 g of cerebral cortex from human (*4*) or sheep (*5*) or squirrel monkey brains (*5*) or from whole brains of mice (*6*) or hamsters (*7*). Each preparation was then treated with octyl-β-D-glucopyranoside and sedimented through a discontinuous sucrose gradient; a band was collected and analyzed for the presence of SAF by negative stain electron microscopy (*1*, *2*).

Scrapie-associated fibrils were present in the three scrapie-infected hamsters, two scrapie-infected squirrel monkeys, one kuru-infected squirrel monkey, and in all six human CJD brain extracts (Table 1 and Fig. 1). These fibrils were not observed in six AD-SDAT cases, four cases of parkinsonism dementia, two cases of ALS, and four other human controls. The SAF were expected but not found in one case of naturally occurring scrapie in sheep, two cases of kuru,

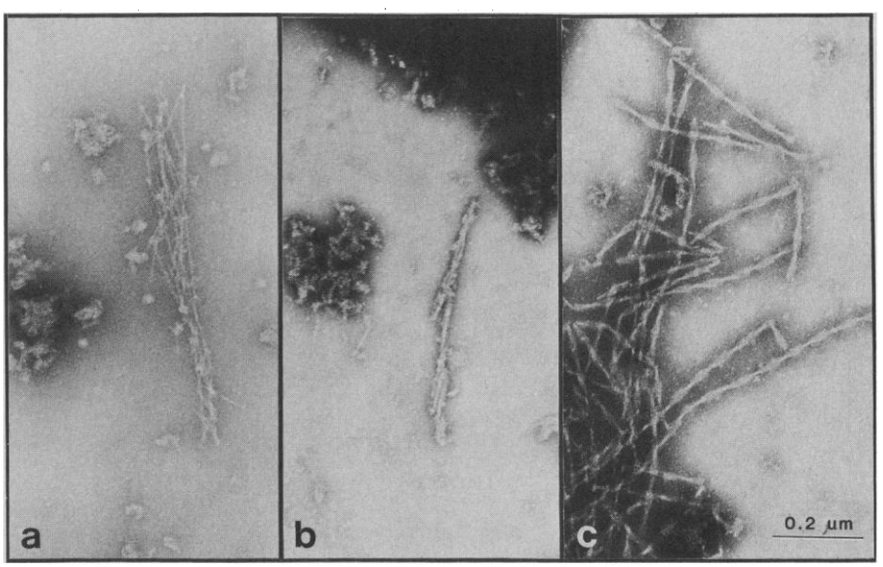

Fig. 1. An example of (a) amyloid fibrils observed in one of the natural CJD cases; (b) SAF from a natural CJD case. The SAF's are a mixture of type I and type II (*2*); (c) PHF observed in extracts from a sporadic Alzheimer case (×90,000). Samples are stained with 3 percent sodium phosphotungstate, *p*H 7.2.

and one case of CJD in squirrel monkey. In summary, SAF have so far been observed exclusively in tissues from individuals infected with "unconventional" slow viruses. However, they have not been observed in every "unconventional" slow virus specimen. Failure to observe SAF in such specimens may have resulted from low infectivity titers either as the consequence of the age and storage history of the sample, or from the chance selection of a less affected locus when sampling from the larger brains, or from intrinsic differences in the maximum titer expressed in various combinations of agent strain and host. For example, maximum brain titers (as LD_{50}, the dose that kills 50 percent of the inoculated population) of the "unconventional" agents of squirrel monkeys are $<10^6$ LD_{50} per gram (*8*), whereas scrapie titers in hamsters reach $>10^{10}$ LD_{50} per gram (*9*).

The specific association of SAF with the "unconventional" slow virus diseases suggests that they are either the etiological agent, a component of the agent, or a product of the pathological process. To further clarify the role of the pathological process in the formation of the SAF, we examined brain extracts from several mouse model systems in which lesions similar to those found in scrapie had been induced experimentally.

Two chemical agents, cuprizone (*10*) and triethyltin (*11*), were used to mimic pathological changes seen in scrapie. Mice fed a diet of 0.5 or 0.75 percent cuprizone in their meal for 8 weeks developed extracellular vacuolation and spongiosis, gliosis, and astrocyte hypertrophy similar to that produced by some mouse-adapted scrapie strains (*12*). Vacuolation was most severe in the cerebellar white matter. Other mice given triethyltin sulfate (30 mg/liter) in their drinking water for 2 weeks showed most prominent vacuolation in the cerebral white matter. SAF were not observed in any of the three cuprizone- or four triethyltin-treated mice (Table 2).

Models of viral-induced spongiosis were also examined for SAF. The M9 mutant Semliki Forest virus was plaque-purified three times in BHK-21 cells. Mice (C57B1/6J) were infected intraperitoneally with 10^2 plaque-forming units (PFU) of stock virus. Some animals were killed 7 days after inoculation when they showed acute clinical signs, including wasting and hind leg paralysis. These animals showed severe inflammatory reaction and neuronal damage in the brain and spinal cord (*13*). Other animals were killed 2 weeks or 2 months after inoculation. These mice showed spongiform lesions of varied size and distribution in gray and white matter (*13*). The model of murine retrovirus–induced spongiform polioencephalomyelopathy was also analyzed for SAF. Mouse neurotropic retrovirus, Lake Casitas strain, was carried in a persistently infected line of SC-1 cells. Virus (10^2 XC PFU) was inoculated intracerebrally or intraperitoneally into newborn (within 24 hours of birth) random bred Swiss-Webster mice (Charles

Table 1. Presence of SAF, PHF, and amyloid fibrils in central nervous system disease. Cerebral cortex or whole brain (0.5 to 3 g) was homogenized and centrifuged to yield a crude synaptosomal mitrochondrial pellet that was then subjected to discontinuous sucrose gradient centrifugation (*1, 2*). The gradient fractions were diluted and processed for electron microscopy (Philips EM 202 or EM 300 electron microscope at 80 KV at ×28,540 through 10× binoculars). Routinely, ten squares of the grid for each sample were examined for SAF-like structures, paired helical filaments (PHF), and amyloid fibrils. Each grid was scored as positive (at least one—SAF, PHF, or amyloid fibril—clearly distinguishable); questionable (SAF, PHF, or amyloid fibrils probably present but not clearly distinguishable from other material on the grid), or negative (no SAF-like structures, PHF, or amyloid fibrils detected). For each questionable or negative grid, a second, and if necessary a third, grid was prepared and examined. Information from all the grids prepared from each sample was collated to determine whether SAF, PHF, or amyloid fibrils were present or absent, prior to the breaking of the code. All control samples were negative on all grids examined.

Host	Disease*	Brains (No.)			
		Total	SAF	PHF	Amyloid
Human	Creutzfeldt-Jakob disease	6	6	2	3
	Alzheimer's (sporadic)	5		5	5
	Alzheimer's (familial)	1		1	1
	Guam Parkinson-dementia complex	4		1	
	ALS with dementia	1			
	ALS without dementia	1			
	Guam Chamorro native	3		2	
	Schizophrenia	1			
Squirrel monkey	Creutzfeldt-Jakob disease	1			
	Kuru	3	1		
	Scrapie	2	2		
	Alzheimer's inoculated (normal)	1			
	Uninoculated	2			
Sheep	Scrapie (natural)	1			
	Normal	1			
Hamster	Scrapie strain 263K	3	3		
	Normal brain inoculated	2			
Mouse	Scrapie strain 139A	2	2		
	Alzheimer's inoculated (normal)	2			
	Normal uninoculated	2			

*Forty-four specimens were coded in two series identified only as containing human and animal models of slow virus disease, other CNS diseases, and controls in unspecified numbers. Because some of the small animal brains could be identified by appearance, the samples were coded a second time before delivering them to the electron microscopist.

Table 2. Absence of SAF in brains exhibiting chemical- or virus-induced vacuolation.

Mouse strain	Cases*	Inducing agent	Pathology	SAF
C57B1/6J	3	Cuprizone	White matter vacuolation	None
Compton white	4	Triethyltin	White matter vacuolation	None
C57B1/6J	4	Semliki Forest virus, chronic	Gliosis with minimal vacuolation	None
C57B1/6J	2	Semliki Forest virus, acute	Gliosis and inflammatory infiltrates with minimal vacuolation	None
Swiss-Webster	4	Neurotropic retrovirus	Spongiform vacuolation of cerebral cortex and spinal cord	None
C57B1/6J	4	Normal brain, inoculated		None
C57B1/6J	4	Scrapie strain (139A affected)	White matter vacuolation	All four

*Twenty-seven specimens were coded in a single series identified as containing chemical and virological controls as well as normal and scrapie-infected mouse brain homogenates in unspecified numbers.

River Laboratories, Cambridge, Massachusetts). Mice showed clinical signs 90 to 100 days after inoculation. Noninflammatory spongiosis and gliosis were seen in spinal cord, brain stem, and cerebral cortex (*14*). Scrapie-associated fibrils were not observed in mice chronically or acutely infected with Semliki Forest virus, nor in mice infected with neurotropic retrovirus (Table 2).

The absence of SAF in these chemical- and viral-induced models of CNS vacuolation and gliosis corroborates other evidence that SAF are not an obligatory ultrastructural feature of spongiform pathology or gliosis. Scrapie-associated fibrils have been observed in the spleens of scrapie- and CJD-infected animals, an organ in which no pathological change has been reported (*3*). Scrapie-associated fibrils have also been observed in brain extracts of scrapie-infected animals before the appearance of clinical signs and histopathological change (*15*); this observation suggests that SAF may be causal agents of the subsequent pathology.

In some of the CJD samples we also observed amyloid fibrils or PHF (or both) along with the SAF (Table 1 and Fig. 1). The three fibrils were distinguishable from each other (*15, 16*). The SAF had a characteristic morphology of two short straight filaments, each 4 to 6 nm in diameter and helically wound around each other, giving a total diameter of 11 to 14 nm and a repeat of 40 to 90 nm. They were observed free on the carbon coat or enmeshed in protein-like material or associated with membrane-like structures. The amyloid fibrils had a total diameter of 4 to 8 nm with a repeat of 35 nm and were observed in small clumps dispersed on the grid (*16*). The PHF were seen as long tangled structures or as short pieces 16 to 18 nm in diameter, narrowing every 70 to 80 nm to 10 nm; they were similar to those described (*15, 17, 18*). Amyloid was also observed in all the AD-SDAT samples. In contrast, no PHF, amyloid fibrils, or SAF were observed in any of the Alzheimer-inoculated animals. Paired helical filaments were observed in one of the parkinsonism dementia specimens and two control specimens from the susceptible Chamorro population of Guam. Paired helical filaments and neurofibrillary tangles have also been observed in normal Chamorros by others (*19*).

A good correlation (Table 3) exists between the ultrastructural and the neuropathological findings in most human cases characterized by abnormal fibril deposition. The electron microscopic examination of fibril type may be a more sensitive indicator of involvement than histopathology. Some combinations of scrapie agent and mouse strain also produce CNS amyloid (*20, 21*). We have, as in the CJD cases discussed above, also observed both amyloid fibrils and SAF in these amyloid-producing scrapie combinations (*16*). Our ability to distinguish SAF, amyloid, and PHF in the presence of each other demonstrates the utility of ultrastructural analysis in the diagnosis of these diseases.

Table 3. Correlation of histopathology with ultrastructural findings. Summary of the human cases only from a coded series of 15 brains in a single experiment identified as containing human slow virus–infected and control brains in unspecified numbers.

Neuropathological changes			Ultrastructural fibers			NIH code
Spongiosis	Neurofibrillary	Plaque	SAF	PHF	Amyloid	
			Creutzfeldt-Jakob disease			
+	−	−	+	−	−	75-175
+	−	−	+	−	−	78-678
+	−	−	+	+	−	71-812
+	−	−	+	−	+	75-128
+	−	−	+	−	+	60-124
+	+	+	+	+	+	77-479
			Senile dementia of the Alzheimer type			
−	+	+	−	+	+	73-178
−	+	+	−	+	+	82-320
			Amyotrophic lateral sclerosis with dementia			
+	+	−	−	−	−	70-572
			Schizophrenia			
−	−	−	−	−	−	77-726
			Cancer			
−	−	−	−	−	−	81-161

The SAF do have certain morphological similarities to amyloid, which suggest a possible precursor product relationship between the two structures. Other than the "unconventional" slow virus diseases, CNS amyloid plaques are only detected in normal, aged brains, where they are found in low numbers, and in AD-SDAT where senile plaques are the principal pathological finding. However SAF were not observed in the brains of AD-SDAT patients containing abundant amyloid. This suggests that SAF are not just another morphological manifestation of amyloid.

Scrapie-associated fibrils are observed exclusively in naturally occurring and experimentally induced "unconventional" slow virus diseases. They are seen preclinically and in organs unaffected by pathology and are therefore more likely to be causal agents than products of pathology. Infectivity studies indicate a correlation between SAF and agent titer (*22*). The time of appearance and quantity of SAF in brain and spleen coincides with a rise in infectivity within these organs (*15, 23–25*). Highly purified fractions of infectious scrapie obtained by Diringer *et al.* (*26*) are highly enriched for SAF. It is possible that SAF may be the etiologic agent of these diseases. If so, SAF represent a new class of filamentous animal virus (*27*).

P. A. MERZ
Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York 10314
R. G. ROHWER
Laboratory of Central Nervous System Studies, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, Maryland 20205
R. KASCSAK, H. M. WISNIEWSKI
Institute for Basic Research in Developmental Disabilities
R. A. SOMERVILLE
A.R.C. and M.R.C. Neuropathogenesis Unit, Edinburgh, Scotland
C. J. GIBBS, JR., D. C. GAJDUSEK
Laboratory of Central Nervous System Studies

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4. *Human material.* Postmortem brains, except for four sporadic AD and one ALS without dementia originated from the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) collection and were verified by neuropathological examination of Formalin-fixed material. The four sporadic AD brains originated from the collection at the Institute for Basic Research and were diagnosed as AD by clinical record and neuropathological examination of Formalin-fixed material. Neurofibrillary tangles and neuritic amyloid plaques were ob-

served in Bodian-PAS (periodic acid–Schiff) stained sections. The ALS case without dementia originated from the ALS Center of St. Vincent's Hospital, New York City, and was supplied by Dr. Donnenfeld.
5. *Sheep and squirrel monkeys*. Normal and natural scrapie sheep brains were from the NINCDS collection of frozen brains. Squirrel monkey brains also originated from the NINCDS collection. Squirrel monkeys were inoculated with (i) tissue from a human case of CJD; (ii) blind passage tissue from a squirrel monkey inoculated with tissue from human AD; (iii) tissue culture explants of two human kuru cases; or (iv) a multiple passaged isolate of scrapie (NIH strain C506). In addition, a multiply passaged chimpanzee kuru isolate was transmitted by feeding to a squirrel monkey. All squirrel monkeys inoculated with unconventional agents were clinically affected when killed.
6. *Scrapie mice*. Female C57B1/6J mice, 8 weeks old, were inoculated intracerebrally with 0.03 ml of a 1 percent mouse brain homogenate from either normal or scrapie strain 139A clinically affected C57B1/6J mice. At the time of clinical disease (4 months) in the scrapie mice, all were killed by cervical dislocation. The brains were removed and frozen at −70°C until processed for SAF. Strain IM/DK mice were inoculated intracerebrally at 8 weeks of age with 0.03 ml of a 1 percent brain homogenate from a postmortem specimen from the brain of an AD patient. Two mice were killed, one at 14 months and the other at 18 months after inoculation. There were no clinical signs, and the routine histology was normal.
7. *Hamsters*. Outbred weanling female golden Syrian hamsters (Charles River–Lakeview Hamstery) received 0.05 ml of either a 10 percent suspension of normal brain or a 10 percent suspension of agent (strain 263K)–affected brain. During the terminal stage of the disease (2 months after inoculation), infected and control animals were killed by CO_2 asphyxiation. The brains were immediately removed and frozen in dry ice.
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25. Scrapie-associated fibrils have been detected in individual spleen extracts of C57B1/6J mice 50 days after an intracerebral inoculation of scrapie strain 139A and 25 days after an intraperitoneal inoculation of a 0.1 percent spleen homogenate prepared from scrapie strain 139A mice.
26. H. Diringer, H. Gelderblom, H. Hilmert, M. Ozel, C. Edelbluth, R. H. Kimberlin, *Nature (London)* **306**, 476 (1983).
27. Since our first publication of the SAS structure in 1981 (*1*), SAF has been isolated in three additional laboratories (*2, 26*, this report). More recently S. Prusiner *et al.* have also reported [*Cell* **35**, 349 (1983)] a rodlike structure isolated from scrapie-infected hamster brains by a method which should produce SAF. These rods have the larger diameter (25 nm) twists and featureless appearance expected when SAF are visualized by the method of rotary shadowing that they used. Negatively stained images of their preparation have the diameters of SAF (11 to 20 nm) and twists; several micrographs indicate that the rods are composed of two filaments. This suggests that the structure described is also SAF.
28. We thank Dr. Richard Kimberlin for providing scrapie strains 139A and 263K; Dr. Alan Dickinson for providing the IM/DK mice; Dr. Greg Atkins for providing the M9 mutant strain of Semliki Forest virus; B. R. Brooks for providing the neurotropic retrovirus, Lake Casitas strain; and our colleagues for their support and encouragement. Supported in part by AGO4220.

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Scoliosis in Chickens: Responsiveness of Severity and Incidence to Dietary Copper

Abstract. *The severity and incidence of spinal lesions were manipulated in a line of chickens susceptible to scoliosis by varying their dietary intake of copper. A decrease in expression of the lesion was related to increased intake of copper. The change in expression, however, appeared to be related only indirectly to the defects in collagen cross-linking, maturation, and deposition known to be associated with dietary copper deficiency. Thus, a dietary constituent in the range of normal intakes may act as an environmental factor in the expression of scoliosis.*

The etiology of the most common clinical varieties of scoliosis has yet to be established. Although investigators have implicated defects in muscular growth, neural development, and connective tissue proteins (*1*), the specific factors that underlie expression of the lesion are unknown. We report here that manipulation of dietary copper alters the severity and incidence of scoliosis in a line of susceptible chickens derived from White Leghorns (*2*).

Many features of scoliosis in this animal are similar to those observed in humans. For example, the lateral spinal curvature is expressed in the thoracic region, the severity of the lesion is often greater in the homogametic sex, and the curves occur predominantly before sexual maturation (*2, 3*). Moreover, there is increased solubility of skin collagen, elevated urinary hydroxyproline, and altered distribution or properties of collagen in vertebral disks (*1–3*).

We investigated the relation of dietary copper to scoliosis because copper has been established as a cofactor in collagen maturation (*4*). Increased intake of cop-

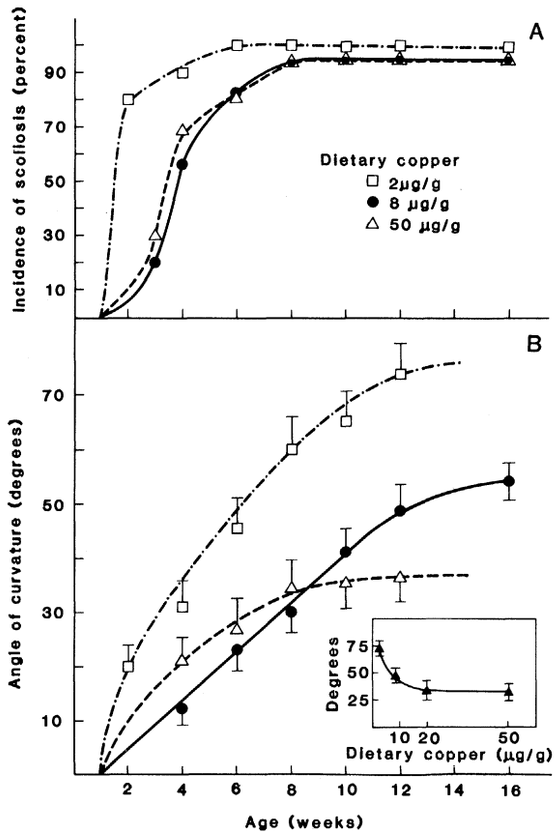

Fig. 1. Effect of dietary copper on the incidence (A) and severity (B) of scoliosis in susceptible chickens. Scoliosis was defined as any lateral curvature in excess of 10° (*9*). Each group contained at least 14 birds (equal numbers of males and females). At 12 weeks the expression of scoliosis exceeded 90 percent in both male and female chicks fed a conventional starter ration containing 6 to 10 µg of copper per gram. The average abnormal curvature was 44 ± 5 degrees in females and 50 ± 4 degrees in males (the homogametic sex in the chicken). The inset in (B) summarizes data for angle of curvature in the various groups at week 12. Values are means ± standard deviations.