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Organelles in Neuroplasmic ("Axonal") Flow: Neurofilaments

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ABSTRACT **The electronmicroscopically conspicuous "neurofilaments" in axons manifest a marked constancy of mutual distance at all levels of a nerve fiber regardless of the wide variation of cross-sectional area along the course of a single axon or between different axons. The filaments conform to the rheological flow patterns of the axonal flow and must be viewed as discontinuous, metastable, and transitory linear assemblies of subunits present in the axonal matrix. The grid-like distribution of the filaments in the matrix suggests properties akin to those of liquid crystals of heterogeneous composition.**

"Axoplasmic flow" refers to the fact (1) that the "axis cylinder" of the mature neuron keeps growing forth throughout life from its base in the cell body, its macromolecular substance being perpetually reproduced there and then conveyed as a cohesive semi-solid mass toward the distal ending of the nerve fiber, where its content is in part dissolved, in part discharged. The whole column moves forward at a standard rate of the order of 1 mm per day, driven by a microperistaltic wave generated in the axonal surface. There is also evidence for a process of much more rapid transfer of substance from center to periphery by means and routes that have not yet been identified [see Dahlström, ref. 2, and recent reviews by myself (3, 4)].

Electronmicroscopic study has revealed that the mitochondria move with the flow (5, 6) and the neuronal microtubules ("neurotubules") advance as single, continuous, and undivided constituents embedded in the axonal column (7), the number of tubular cross sections in single branched axons being the same in the common stem and in the sum of the branches.

In sharp contrast to this numerical invariance of the *neurotubules*, the number of *filaments* varies in direct proportion to the actual local cross-sectional area of the axon. This distinction, added to that of protein composition (8), called for a critical reassessment of the electronmicroscopic features of those two systems.

Objects and Methods were the same as in the preceding article on neurotubules (7): small motor nerve fiber bundles; 150–250 g rats, perfused by buffered glutaraldehyde; postfixed in osmium tetroxide; embedded in araldite or epon; thin-sectioned at consecutive levels; stained in uranyl acetate and lead citrate; sample sections from different levels, microscopically identified as of one and the same axon, photographed at identical magnifications under the electronmicroscope.

On first inspection (Figs. 1 and 2), we were struck (*a*) by the regularity of the spacing between the individual filaments and (*b*) by their being grouped in bundles of parallel lines, often aligned in rows, predominantly in the general direction of the nerve fiber axis, but each bundle following a detailed course of its own, rather sharply set off from the path of its neighbors. As a result, slightly oblique sections show filament bundles in various slants, some transversal, others in lateral profile (Fig. 3).

Measurements of 227 interfilament distances yielded the histogram of Fig. 4, showing a rather sharp peak at 428 Å, i.e., an average domain per filament of roughly 2×10^5 Å^2, or about 500 filaments per square micron. From this rough estimate of average filament density, we then proceeded to a more direct determination of the ratio between the number of filaments and the actual area of axonal matrix occupied by

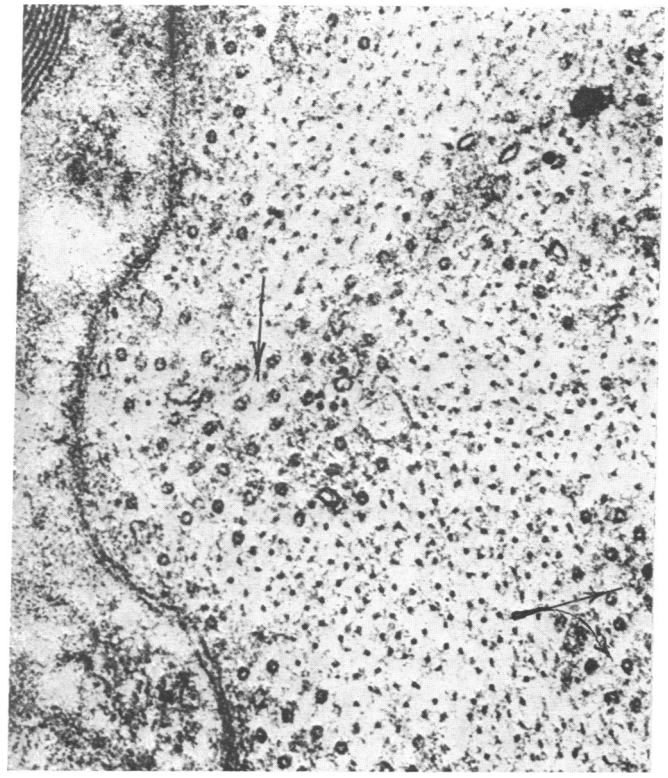

FIG. 1. Axonal cross section with clusters of neurotubules (e.g., at *arrows*) and neurofilaments. ×58,000

This paper is part III of a series on organelles in axoplasmic flow. Part II is ref. 7.

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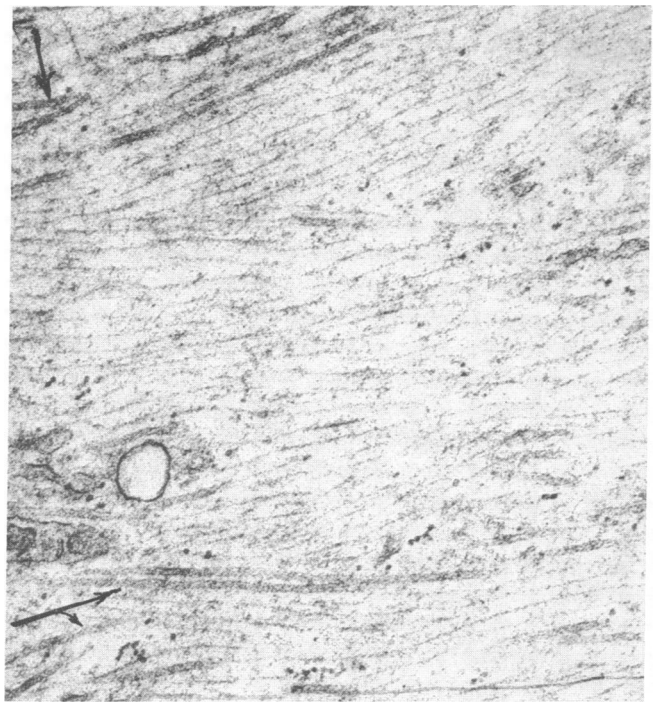

FIG. 2. Longitudinal axonal section with neurotubules (*arrows*) and filaments. ×58,000

them, the latter measured as total axonal cross section reduced by the space (about 15%) occupied by mitochondria, endoplasmic reticulum, and neurotubules.

The diameter of a single axon fluctuates considerably along its axis. This variability extends way beyond the familiar narrowing at each node of Ranvier over all internodal sections. The following laborious counts and measurements were carried out on three single axons, referred to as A, B, and C. Cross sections were measured by planimetry. Filaments were counted either over the entire section (case A), or over a representative unit sampling area (cases B and C); in the latter procedure, the count of the sample unit was then multiplied by the ratio between the cross-sectional area of the axon (corrected downward for the area occupied by mitochondria

TABLE 1. *Proportionality between number of filaments and local axonal area*

Case	Level	Axonal area (μm^2) Total area	Relevant area*	No. of filaments	No. of filaments per μm^2	No. of tubules
A	a	1.84	1.54	340	230	17
	b	1.62	1.36	231	185	18
	c	0.53	0.49	111	260	18
B	a		10.7	1,600	150	
	b		1.7	175	103	
	c		6.3	930	148	
	d		5.5	880	160	
C	a		3.5	650	186	
	b		2.6	570	218	
	c		12.5	2,800	224	

* Axonal cross-sectional area, reduced by cross sections of mitochondria and endoplasmic reticulum.

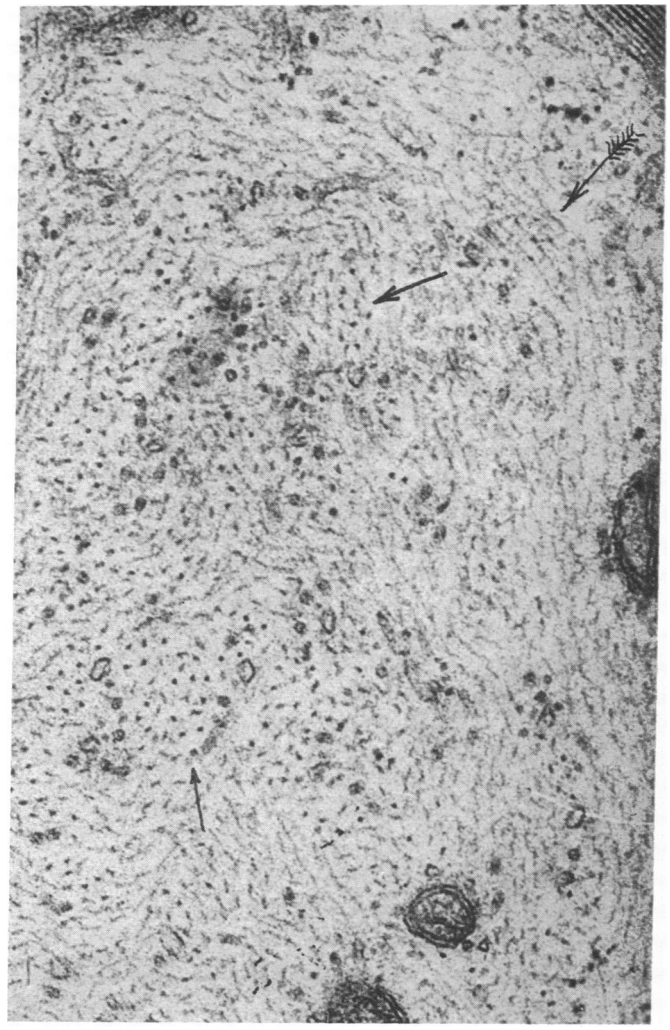

FIG. 3. "Streamlined" fascicles of filaments in transverse (*single arrows*), lengthwise (*feathered arrow*), and oblique courses. ×58,000

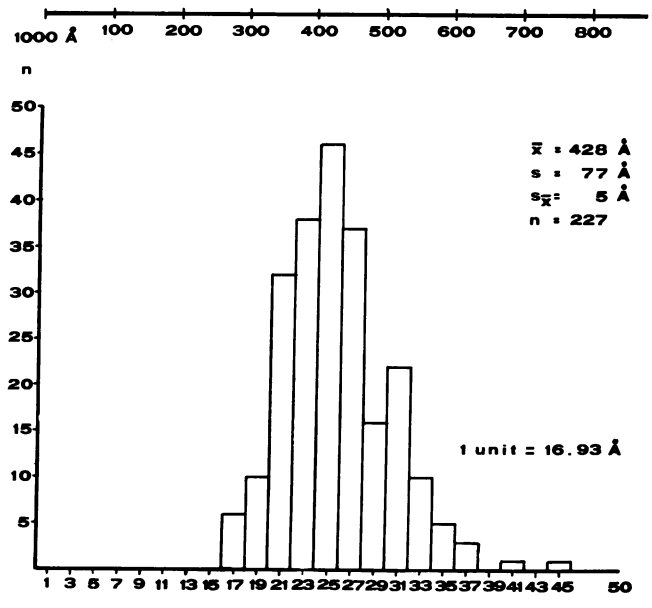

FIG. 4. Histogram of interfilament distances.

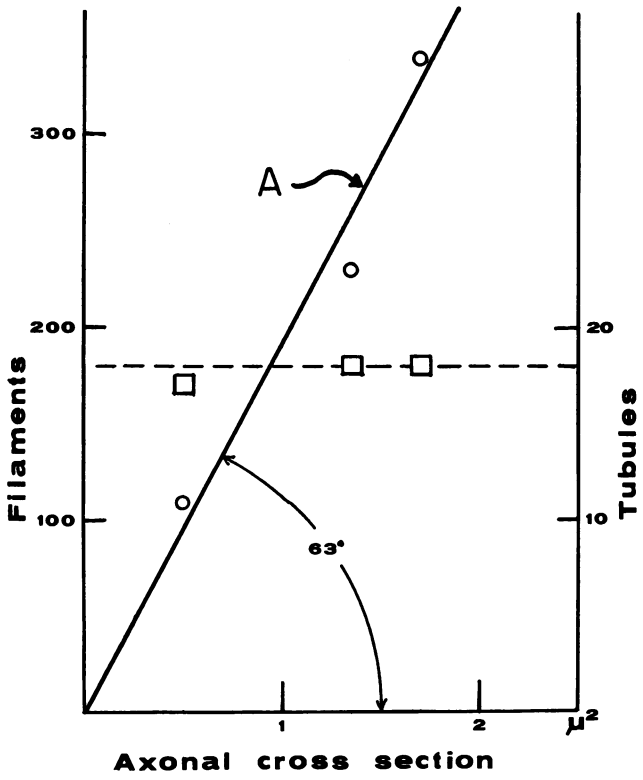

FIG. 5. Plot of number of filaments (*circles*) and tubules (*squares*) against relevant cross-sectional areas of single axon A.

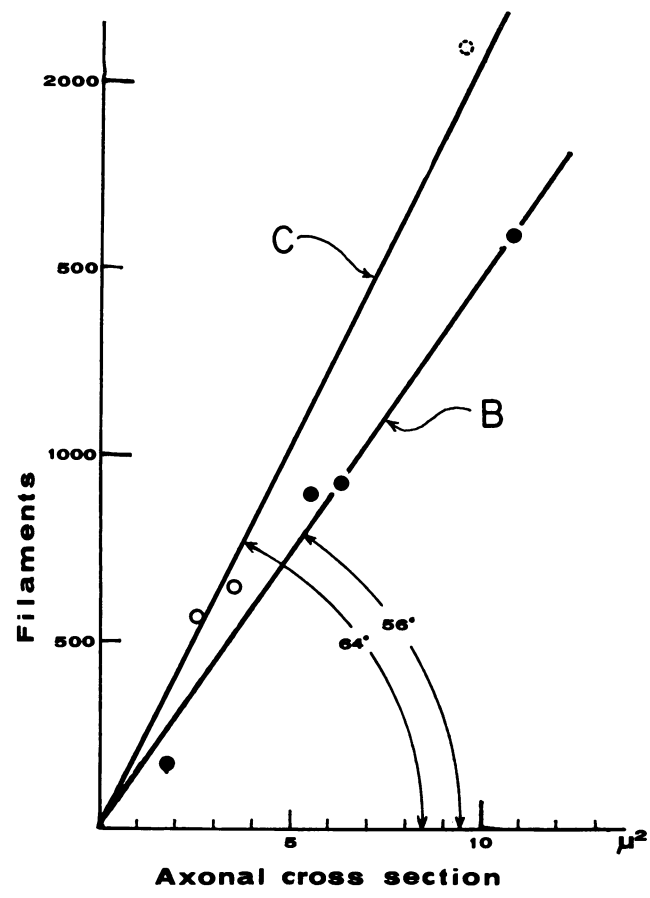

FIG. 6. Plots of numbers of filaments against relevant cross-sectional areas of axons B (*full circles*) and C (*open circles*).

and reticulum) and the sampled area (minus microtubular space). Table 1 gives the respective values.

Case A. The relevant axonal area at level *a* is 3.14 times as large as that at *c*, and the number of filaments counted in *a* is 3.07 times as high as that in *c;* that is, the numbers of filaments are roughly proportional to the areas of matrix they populate. The respective proportionality factors between the three levels are $a:c = 1.25$, $a:b = 0.80$, $b:c = 1.33$, for an average of 1.13. The graph in Fig. 5, plotting number of filaments against corrected axonal areas, illustrates this relation. Contrastingly, the count of neurotubules was essentially the same at all three levels (17, 18, and 18; broken line in Fig. 5) (7).

Cases B and C. [Table 1 and Fig. 6; the point for level *c* of case C, lying beyond the limits of printing space, is represented in the figure by a point (broken contour), both coordinates of which have been proportionately scaled down by one fourth.] Given the uncertainties of sampling error, and errors of planimetry and counting, it is all the more striking that the number of filaments per unit area of matrix, i.e., the domain of matrix belonging to a single filament, has a *near-constant* value regardless of the actual size of the total area in which they are counted. Consider, for example, that the largest area (at level C, *c*) was 7.35 times as large as the smallest one (at B, *b*), and that the former contained 16 times as many filaments as the latter, whereas the average numbers of filaments per unit area in these two extremes differed only by a factor of 224:103 = 2.2, well within the limits of error of the method.

Comparing the three unrelated axons A, B, and C, the combined average of filament density for all ten levels assayed (total number of filaments divided by total relevant matrix area) is 180 filaments/μm^2; the separate values are, for the fully counted case A, 227, and for the extrapolated estimates of B and C, 140 and 209, respectively, thus fluctuating about the total average of 180 by no more than 26%. It is obvious that such a 26% deviation becomes quite insignificant if one compares it with the range of variation of 2500% of the populations of filaments and of the sizes of areas over which it held true. Therefore, the proportionality between any local area of organelle-free axonal matrix and the number of filaments passing through it is of standard value both within a given axon and for axons of different nerves, at least those treated by the same histological procedure. The higher value of filament density (500/μm^2), calculated from Fig. 4, but less comparable because of different osmolarity of the fixing fluid in that case, is still of the same order of magnitude. Figs. 5 and 6 document (*a*) the linear proportionality within each of the three studied axons and (*b*) the essential identity of the proportionality factor for all three, expressed by the near-identity of the slopes, angles being strictly comparable, as all coordinates in Fig. 5 are proportionately scaled down in Fig. 6 by a factor of 5.

The wide variance in the number of filaments among levels proves that filaments are not continuous throughout the length of an axon, but are fragments of limited extent (9, 10), their fractional lengths being a linear function of the local mass of matrix. This is in sharp contrast to the numerical constancy

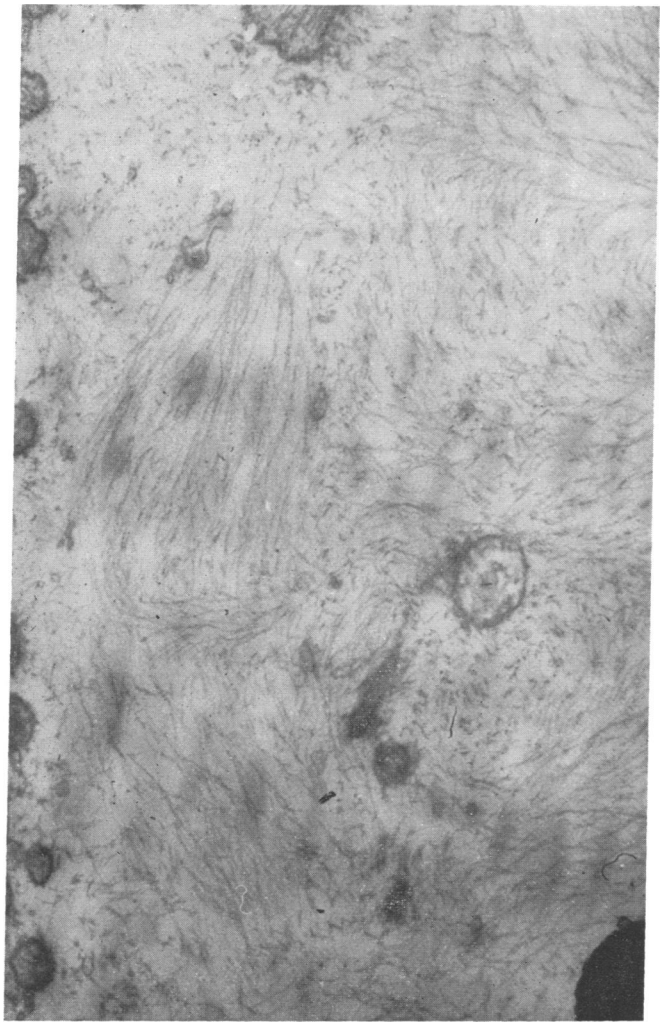

FIG. 7. Electron micrograph of cross section through dammed portion of axon near entrance to chronic constriction. (Specimen from old experimental series with simple osmic acid and methacrylate processing, hence greater distortion of detailed fiber courses.)

and continuity from cell body to ending of the neurotubules (7) (see Fig. 5, broken line).

Unexpectedly, the lines of all three graphs pass through the zero point. This is so uncommon for biological phenomena that it raises the question of how faithfully the visual image of "neurofilaments" preserved *in situ* corresponds to the actual physical state of axoplasm in the *living* nerve. We avoid the term "artifact", because any static picture of a "fixed" living process deserves that designation. We consider a given pattern of aggregation, orientation, and array in space of fine structural detail after its histological processing as merely a clue for its counterpart in the living state modified by the processing. In this sense, the evidence for the structural identity of the neurotubules is compelling, whereas the evidence for neurofilaments is not.

Our electronmicrographs confirm all major features ascribed to neurofilaments in the literature: diameter of 8–100 Å; linear strings of globular subunits with regular periodicity of 120 Å; lower electron density in the axis, simulating a lumen (equally well explicable by the metallic stain coat of the wall barring penetration of stain to the inside); and signs of zig-zagging, ascribed to helical twisting (but in contradistinction to clearly defined spirals (11), referable to optical side effects). Plainly, proteinaceous subunits (8) preexist in the neuronal matrix, which, under the appropriate conditions, aggregate into filaments of the described form of order. Their parallel orientation within a given bundle (Figs. 1–3) indicates vectorially oriented dynamics in their common matrix, and change in orientation between neighboring skeins (Fig. 3) reveals sharp local divergencies in those force fields. The relatively constant spacing of the filaments and their alignment in grids suggest a paracrystalline substructure (macrolattice pattern) of the matrix, subject to distortion, such as has been deduced for other fine structural patterns (12). The filaments thus appear as fibrous crystals, each commanding a concentric domain of standard radius, with the local mass of matrix at each level determining the number of domains that can be accommodated there.

This unifying hypothetical interpretation of the neurofilaments becomes almost a postulate in the context of the principle of axonal flow. As the moving axonal column narrows at each node and widens again past the bottleneck, numerical proportionality to local caliber could be preserved only if stretches of filaments in the internodes are continually dissolving at their front ends and reconstituted at their rear ends. In other words, filaments are not only discontinuous, but of very transitory existence: their micrograph represents no stable structure, but just the momentary configuration of flux of molecular assembly and redispersion. This redefines filaments from static architectural fixtures to markers of the dynamic force patterns of their matrix; and since that matrix is in steady flow, they can be viewed as embodied *stream lines*.

This view is borne out by their convoluted and whorl-shaped configurations in regions of damming of axonal flow, either natural, as at nerve terminals, or at the upstream side of artificial constrictions. A fuller documentation and discussion of this phenomenon is in preparation. By way of example, Fig. 7 is an axonal cross section proximal to such a constriction, showing longitudinal, transversal, and oblique bundles of filaments criss-crossing the field, always in groups, analogous to the pattern of Fig. 3 in a near-terminal region of a normal axon. The line systems in both correspond to known patterns of rheological turbulence, such as eddies.

In conclusion, we put forth the concept that the neurofilaments, prominent in electronmicroscopic view, are metastable linear aggregates of anisodiametric subunits of ubiquitous occurrence in the axonal matrix, arrayed along the shear lines of axonal flow, much as small rod-like molecules line up in streaming double refraction (13). In addition, however, we must assume that the aggregation proceeds from nucleation points set apart by "forbidden spaces", analogous to giant "lattice constants"; either flowing axoplasm shares properties with inorganic liquid crystals or, after the recruitment of a filament, the critical conditions for the recruitment of another cannot arise except beyond the competitive range of the former. Whether the transition from the dispersed into the metastable state of electronmicroscopic fibrillity is due to the preservation or occurs reversibly incessantly in the living axon, is best left to the future for decision. For a promising lead pertinent to our conjectures about filament patterning, we cite a phenomenon of potential bearing recently described for an inorganic system (14). When a melted mixture of tungsten metal and uranium dioxide was allowed to solidify unidirectionally,

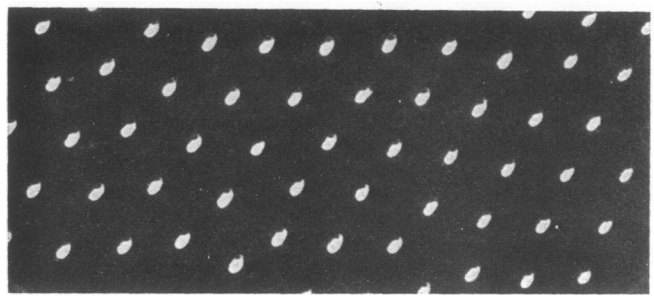

FIG. 8. Distribution of tungsten fibers after unidirectional solidification of a melt of tungsten and UO_2 (see text). [From ref. 14].

the consolidated mass, shown in Fig. 8 in cross section normal to the "flow" axis, consisted of a matrix of UO_2 in which tungsten fibers of uniform diameter had formed in a neat grid of parallel lines along a system of intersecting planes at regular interspaces (3–6 μm). Since all these criteria, except for the larger dimensions involved, duplicate the features of the filament pattern in the flowing axonal matrix, the possibility of a common dynamic principle of directional and distributional self-ordering in multicomponent assemblies suggests itself.

Neither our findings nor our conclusions pertaining to nerve fibers *in situ* are directly comparable to observations on fixed *extruded* axoplasm (15) or on preparations in which the regularities described by us might have been distorted or abolished by alterations in the histological processing. Similarly, comparisons between varying neurofilament-to-neurotubule ratios (16) suffer from the disparity of the lumped categories; yet the qualitative conclusion that the number of neurotubules is stabilized early in development, whereas the population of filaments keeps mounting with the growing caliber of the axonal matrix, is well in accord with our data. On the other hand, an earlier suggestion (17), ascribing to the interfaces between neurofilaments and matrix a guiding function in molecular transport, is now ruled out by the instability and discontinuity of the presumed communication lines.

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