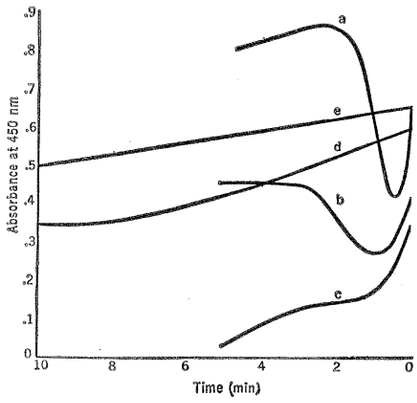

Fig. 3. (*a* to *d*) Action on *M. lysodeikticus* of blood taken from insect injected (*a*) 5 days (compare with Fig. 1*d*), (*b* and *c*) 13 days, and (*d*) 18 days previously with 0.05 ml of 2 percent lysozyme solution. (*e*) Action on *M. lysodeikticus* of blood taken from insect injected 18 days previously with 0.05 ml of 1 percent lysozyme solution.

result in a reaction mixture containing about 0.012 mg of lysozyme, approximating the 0.01 mg of enzyme used in the reference assay (Fig. 1*a*).

Adult male cockroaches were injected with 0.05 ml of 1 or 2 percent lysozyme in 0.5 percent NaCl solution and examined at intervals of several days. Blood (15 to 20 μl) was rapidly withdrawn from the chilled insect and mixed with 2.6 ml of bacterial suspension; the mixture was analyzed in a Beckman linear recording spectrophotometer at a wavelength of 450 nm. The reaction obtained with blood withdrawn 5 days after injection of 2 percent lysozyme (Fig. 3*a*) duplicates in all essentials that of lysozyme in a mixture of normal blood and micrococci (Fig. 1*d*). Blood withdrawn 13 days after injection of 2 percent lysozyme also reproduces this curve (Fig. 3*b*), although indications of lower lysozyme concentration are seen in this and still more in another specimen (Fig. 3*c*). Activity is still evident 18 days after injection (Fig. 3*d*), even after injection of 0.05 ml of only 1 percent lysozyme (Fig. 3*e*). It may then be concluded that the indicated activity is due to the presence of residual lysozyme.

The addition of 20 μl of normal blood to a reacted mixture of lysozyme and *M. lysodeikticus* results in an immediate and sharp increase in absorbance, which continues as a gradually rising curve (Fig. 2*b*). In view of this and of the sharp increase that follows the initial reduction in absorbance in a suspension of the bacteria to which has been added either normal blood and then lysozyme (Fig. 1*d*), or blood from a cockroach injected with lysozyme (Fig. 3*a*), it appears that lysis of the bacterial cell wall enables the lysed bacteria to react with the blood to produce highly absorbing products. Thus, lysis must first begin; but since the change in absorbance does not equal that produced by lysozyme acting on the bacteria alone, it seems that the bacterial products are already reacting with the blood before lysis is completed. It is therefore not feasible to assay the lysozyme concentration in the presence of blood. The foregoing phenomenon may explain the reaction of certain normal bloods with the bacteria (Fig. 2*a*). The blood of *P. americana* is weak in native lysozyme and *p*H 3.5 is the optimum for activity of this enzyme (*7*). With a low concentration and less than optimum *p*H, lysozyme could be conceived as having a weakly lytic action on the bacteria, which is made all the more cryptic by the competing absorbance of the developing reaction between bacterial cell products and blood; hence the lack of a decline in absorbance and the delayed increase. We have no proof, however, that the observed reaction is the consequence of lysozyme action.

It is interesting that the injected lysozyme should persist in the insect for a considerable time, for notwithstanding Malke's view (*3*) that the egg-white lysozyme used was identical with the insect's lysozyme and therefore incapable of causing a foreign body reaction, Powning and Irzykiewicz (*7*) have shown that they differ. The observed evidence of its toxicity in the cockroach (*8*) is probably due to both its difference and its persistence.

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23 September 1968

Preferred Centripetal Conduction of Dendritic Spikes in Alligator Purkinje Cells

Abstract. *Dendritic action potentials in alligator Purkinje cells tend to have a unidirectional preference which favors centripetal over centrifugal propagation. This unidirectional tendency funnels the peripherally evoked dendritic spikes into the lower dendrites and soma of these cells, and it allows the peripheral dendritic branches to operate to a certain extent as partially independent functional units.*

Alligator Purkinje cell dendrites have been shown to generate action potentials near their end terminals (*1*). These action potentials seem to be conducted toward the cell's soma with an average velocity of 30 cm/sec, the conduction velocity being smaller at the periphery (10 cm/sec) than at the main dendritic branches (40 cm/sec). The results to be reported here suggest that the conduction of these spikes is preferentially centripetal although centrifugal dendritic invasion can also occur.

Alligators (*Caiman sclerops*) were anesthetized with pentobarbital and then immobilized with gallamine (50 mg/kg intraperitoneally) and artificially ventilated. The surface of the cerebellar cortex was stimulated electrically by means of a local electrode which activated a small bundle or "beam" of parallel fibers in the molecular layer (*1*, *2*). Purkinje cells were also activated antidromically through a bipolar electrode (WM) in the white matter near the cerebellar peduncle. The field potentials generated by local (Loc) or white matter (WM) stimulation were recorded by an array of three or four micropipettes filled with 4*M* NaCl and which had an average d-c resistance of 1 to 2 megohm. The micropipettes were placed in a carrier which allowed their tips to be positioned at 200 μ from each other laterally and in the same vertical plane with the height of each electrode adjusted so that they entered into simultaneous contact with the cerebellar surface. This particular interelectrode distance was chosen because the lateral spread of the Purkinje cell dendrite is in the vicinity of 250 to 300 μ, and so the two electrodes would be within one Purkinje cell spread from each other. Each microelectrode was connected to a separate field-effect transistor amplifier whose output was displayed on an eight beam dual-gun oscilloscope.

Field potentials evoked by parallel

fiber stimulation are illustrated in Fig. 1B. The uppermost trace of each group of records came from electrode 1, the second from electrode 2, and so on. With respect to the beam of activated parallel fibers, electrode 1 was in line, electrode 2 was near its peripheral border, electrode 3 was 200 μ lateral to the border of the beam, and electrode 4 was 400 μ lateral. The records were taken first at a 400 μ depth, which corresponds to the Purkinje cell layer (*1*), and then at every 100 μ as the electrodes were withdrawn up to the surface. At 400 and 300 μ local parallel fiber stimulation (Loc) evoked in all electrodes long lasting negative fields with superimposed fast positive-negative spikes. At 200 and 100 μ from the surface the negativity became progressively delayed in tracks 2, 3, and 4 with respect to track 1. The early positive-negative transient (down going arrow) reflects the parallel fiber field (*2*) and is visible from 200 μ and upward in tracks 1 and 2 exclusively. The amplitude of the positive-negative wave is larger in 1 because of its in-line position. At levels above 100 μ, the fields of electrodes 2, 3, and 4 showed a predominantly positive rather than a negative deflection. This indicates the existence, at that level of current sources, to the current sinks created by the activation of deep dendritic spikes as can be assumed by the fact that the time course of the positivity is similar for all four electrodes. In trace 1, the parallel fiber activation is followed by a large negative-positive transient. The latter has been attributed to a spike generated by the peripheral dendrites immediately under the activated beam of parallel fibers (*1*). These records suggest that the superficial dendritic negativity is conducted toward the soma and does not invade antidromically other dendritic branches of the activated cells.

Two other examples of this lack of centrifugal invasion to neighboring dendrites are seen in Fig. 1, C and D. In this case the lowermost record of each set corresponds to the electrode in line with the activated parallel fibers. The others are located 200 and 400 μ lateral to the parallel fiber beam. Records in C and D were taken in different tracks at 100 μ from the surface, and the strength of the Loc stimulus was gradually increased from the top to the bottom set of three records from near threshold for parallel fiber activation through 1.5, 1.7, and 2.0 times threshold. As the stimulus increased, the field potentials increased in amplitude, and the latencies for the in-line negativity and the out-of-line positivity became shorter. It must be noted that, even at near-threshold stimulation (Fig. 1C), the in-line negativity and out-of-line positivity were closely correlated in latency and amplitude. The out-of-line positivity was usually present down to about 150 μ from the surface. Deeper, the fields evoked by Loc stimulation were all negative. In all instances at 200 to 250 μ, the out-of-beam negativity had a longer latency than its in-line counterpart, possibly because it was generated by partial centrifugal invasion

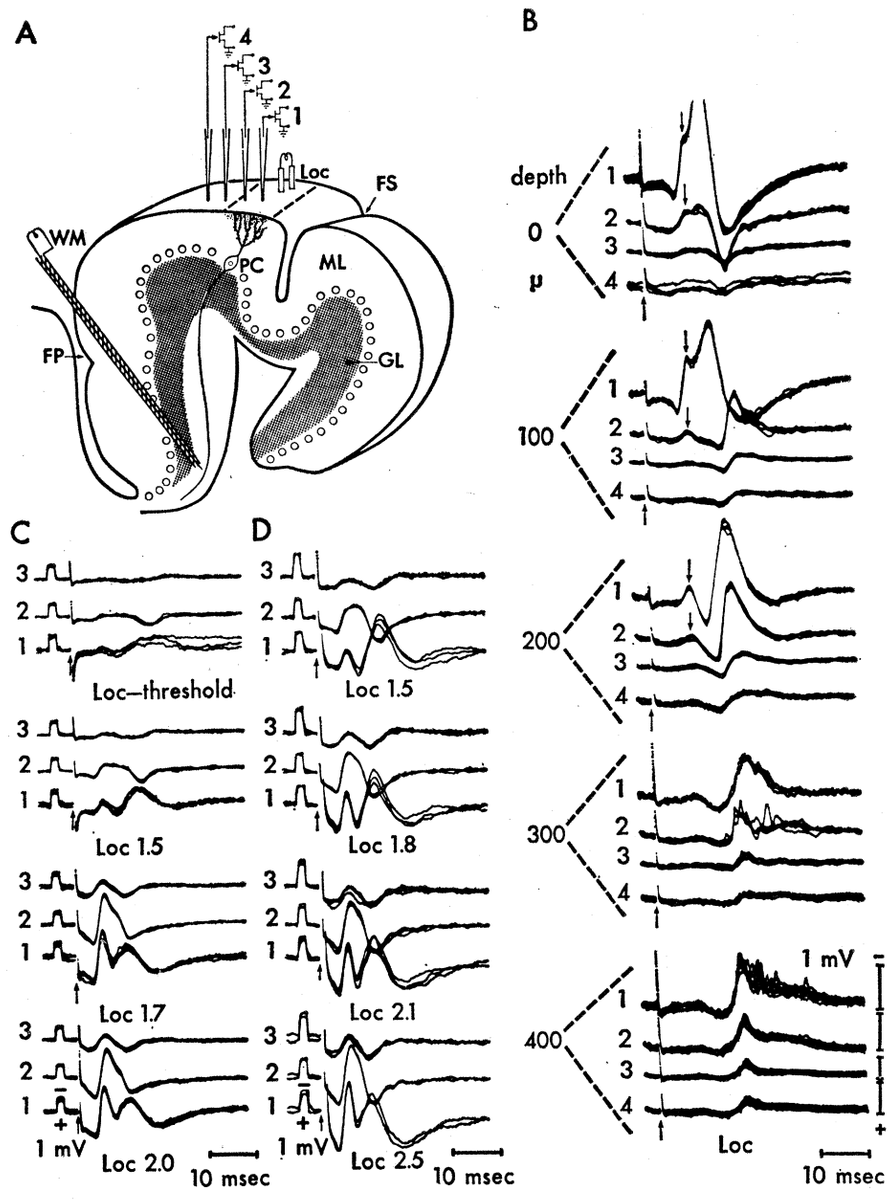

Fig. 1. (A) Diagram of alligator cerebellum and experimental arrangement. *FP*, fissura prima; *FS*, fissura secunda; *GL*, granular layer; *Loc*, local stimulating electrode; *ML*, molecular layer; *PC*, Purkinje cell layer; *WM*, white matter stimulating electrodes. The recording microelectrodes (1,2,3,4) are introduced simultaneously in the cerebellar cortex at a distance of 200 μ from each other. (B) Set of field potentials recorded by a four-microelectrode array. Each group of four traces was taken at the depth shown to the left of each set of traces. The digit just left of each trace corresponds to the microelectrode arrangement as shown in A. (C and D) Potential field recorded at 100 μ depth from two different tracks with a three microelectrode recording probe. As in B, traces 1, 2, and 3 refer to the position of the microelectrode with respect to the beam of parallel fibers. In C, traces in the uppermost set of records were evoked by a near-threshold Loc stimulation; the other three sets of records by Loc stimulation of increasing strengths. The numbers under each group represent the intensity of the Loc stimulus with respect to threshold. In D, as for C, the stimulus used was slightly higher. Amplitude, polarity, and time calibrations always as marked in the lowermost groups.

from lower dendrites. Such partial centrifugal invasion is demonstrated very clearly in Fig. 1B, trace 2, at a depth of 100 and 200 μ. The latency for the negativity at 200 μ is shorter than for the negativity recorded at 100 μ in the same track, which suggests upward invasion.

A different method of testing the excitability of dendrites is to activate Purkinje cells antidromically by means of a bipolar electrode. Electrical stimulation of the cerebellar white matter at peduncular levels activates mossy and climbing fiber systems as well as Purkinje cell axons. The latter activate antidromically the soma and initial segments of the Purkinje cell dendrites as reported for the cat (*3, 4*). White matter stimulation evokes in the alligator an early negative field seen clearly at 400 μ from the surface, the level of the Purkinje cell layer (Fig. 2A). This early field is followed by a longer lasting negativity which is evoked by the activation of the mossy and climbing fibers and which also resembles the potentials evoked under similar conditions in the cat (*5*). When the recording microelectrode is at levels of 200 μ or less, the early negativity reverses to a sharp positivity. The latter finding suggests that the antidromic invasion of the dendrites does not reach that level (Fig. 2D) (*4*). Records 2B show that Loc stimulation does evoke large negative fields at these levels (Fig. 2B). The amplitudes of the negative components of the antidromically and of the locally evoked fields are plotted against depth in Fig. 2C for another set of records.

The sum total of these results suggests that, in alligator Purkinje cells, the action potentials generated in the peripheral dendrites have a centripetal conduction tendency which would funnel these action potentials toward the cell's soma (Fig. 2E). However, since an antidromic invasion of the lower dendrites is strongly suggested (Fig. 2A) and an out-of-line negative field can be occasionally observed (Fig. 1B), the unidirectionality of the dendritic spikes may not be absolute and, as demonstrated by Hild and Tasaki in neuronal tissue culture (*6*), activation of one dendrite may lead to partial invasion of its neighbors.

Several consequences of these findings can be mentioned. The fact that Purkinje cell dendrites generate spikes implies that dendrites may operate as relatively independent units whose activity would be a function of the synaptic impingement acting upon them and of the functional state of its neighboring branches. Once a particular dendritic branch reaches its threshold, its action potential would be transmitted toward the soma, but not necessarily to other dendritic branches, although the neighboring branches may be depolarized by electrotonic spread. In this manner each dendrite could operate without disturbing other branches, by generating within them centrifugal action potentials. The lack of dendritic invasion that follows WM stimulation implies that the axosomatic action current is not sufficient to depolarize the dendritic tree to firing level. This low safety factor can be explained by postulating a high threshold for peripheral dendrites or a high load for the action currents at dendritic levels, or both. The first assumption is strengthened by the fact that the spike conduction in these dendrites is of a noncontinuous character (*1*), and that in order for these spikes to be evoked the strong synaptic depolarization produced by the activation of the parallel fiber beam is required. Finally, another factor which must be kept in mind regarding unidirectionality in dendritic conduction is the impedance mismatch produced by the changes in the internal longitudinal resistance of the dendritic tree as the diameter of the core conductors becomes smaller in its periphery.

The existence of neurones with multiple sites for spike origin raises interesting theoretical questions (*7*). Given the results presented, we feel that alligator Purkinje cells and probably many other nerve cells may have to be considered as highly complex units able to attain a vast number of dynamic states which would lead to the generation of a large variety of functional patterns.

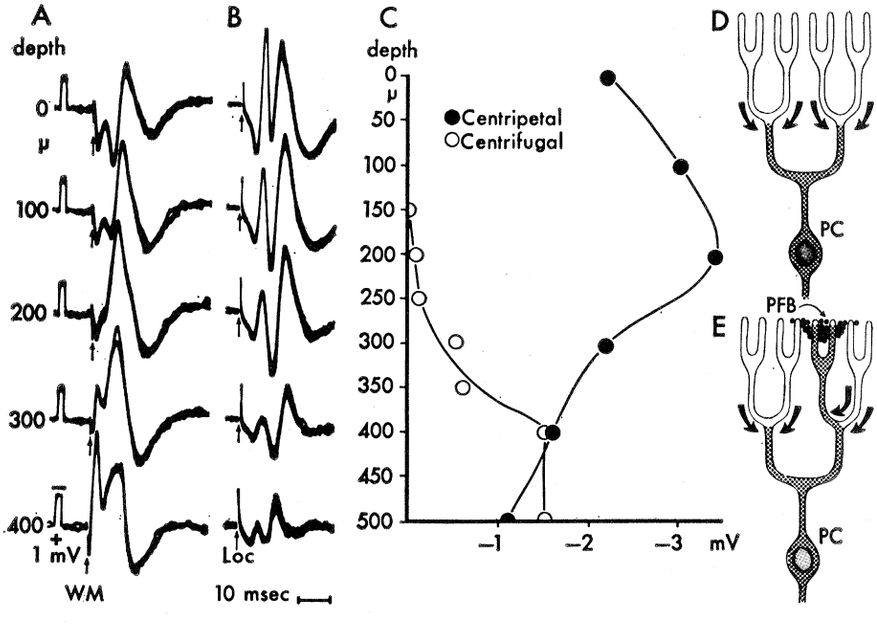

Fig. 2. (A and B) Field potentials evoked by alternate Loc and WM stimulation and recorded at different depths. In A the early positive-negative transient recorded at 400 μ is evoked by the antidromic invasion of Purkinje cells. Their field is reversed to a positivity at depths of 200 μ and above. The long lasting negative-positive field which follows in time is evoked by the activation of the mossy and climbing fiber afferent system. In B, Loc stimulation activates a bundle of parallel fibers (first sharp positive-negative field at all levels), after which a long lasting negative-positive wave is observed, which corresponds to the generation of spikes in the dendrites of the Purkinje cells (*1*). (C) Amplitude of the antidromic negativity at different depths (open circles) and of the negativity evoked by Loc stimulation (closed circles); ordinate, depth in microns from the cerebellar surface; abscissa, amplitude of the field in millivolts. (D and E) Diagrams of alligator Purkinje cells (*PC*) to illustrate the current flow between different portions of this neurone after WM and Loc stimulation. In D, the dotted area represents the regions of the neurones which act as a current sink following antidromic invasion by WM stimulation. The areas in white act as current sources. Arrows represent the direction of current flow. (E) As D, but the cell is being activated by Loc stimulation. The dendrites immediately under the beam of activiated parallel fibers (*PFB*) act as current sinks and evoke a negativity (dotted area) as the action potentials are conducted downward toward the axon.

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17 October 1968

Microtubules in Brain Homogenates

Abstract. *Microtubules from neurons are preserved in homogenates of mammalian brain by medium containing organic solvents at acidic* p*H. By means of negative staining and electron microscopy, the relative concentration of microtubules in suspensions can be assayed. Microtubules from brain have a filamentous substructure.*

Microtubules observed in axons and dendrites appear to be minute tubes about 250 Å in diameter and of indefinite length. They are morphologically similar to microtubules in other tissues, such as mitotic apparatus (*1*), juniper root (*2*), and a protozoan (*3*), but their function is not understood. Previous techniques of preparing homogenates of brain have not permitted the preservation of microtubules in vitro. I now describe a method of preserving microtubules in brain homogenates and for determining their concentration in suspensions.

The "crude mitochondrial" fraction from homogenates of whole brain or cerebral cortex in 1*M* hexylene glycol (*4*) at *p*H 6.4 in 0.01*N* potassium phosphate buffer (*5*) was collected by centrifuging at 10,000*g* for 20 minutes after a preliminary centrifugation at 1000*g* for 10 minutes to remove nuclei and tissue fragments. The pellet was either fixed with 3 percent glutaraldehyde, treated with osmic acid, dehydrated, embedded in plastic, thin-sectioned, and positively stained, or it was resuspended in medium and fixed with glutaraldehyde for negative staining with phosphotungstate and then examined by electron microscopy. All experiments were performed at room temperature.

Microtubules in crude mitochondrial suspensions were assayed by counting the number of microtubules identified on negatively stained grids scanned in the electron microscope. Grids for assay were coated with Formvar and carbon, and a drop of suspension was applied for 15 seconds. The excess was drained off with filter paper, and drops of dilute serum albumin and 2 percent potassium phosphotungstate were applied and drained immediately. Grids from all experiments were first coded; then the microtubules were counted without knowledge of their source. The accuracy of counting and sensitivity of the assay were tested by preparing a microtubule-rich suspension of crude mitochondria from whole rat brain, homogenized in 1*M* hexylene glycol at *p*H 6.4, which was fixed 30 minutes after death of the animal. This suspension was diluted with a crude mitochondrial fraction from rat brain homogenized in 0.01*N* phosphate buffer only at *p*H 7.4 and aged overnight at 4°C. The diluent contained no identifiable microtubules but provided a background of mitochondria, synaptic endings, and membrane fragments among which microtubules must be identified in the assay technique.

The relative importance of hexylene glycol and lowered *p*H in preserving microtubules was tested in four experiments in which the crude mitochondrial fraction from a homogenate in hexylene glycol, at *p*H 6.4, was transferred to control media providing either or none of the experimental alterations. These suspensions were fixed 2 hours after death of the animal, 1½ hours after exposure to the changed media, and were assayed by the negative stain method.

Microtubules from brain homogenate embedded in plastic and stained positively appeared identical to microtubules observed in thin sections of neurons. The neuronal origin of the microtubules was confirmed by the occasional identification of a partially disrupted axon that contained microtubules (Fig. 1A). Microtubules were more frequently free in the homogenate (Fig. 1B), in groups or singly. Some of these microtubules probably originate from glial cells; but, because in thin sections of brain most of the microtubules are neuronal, it is believed that

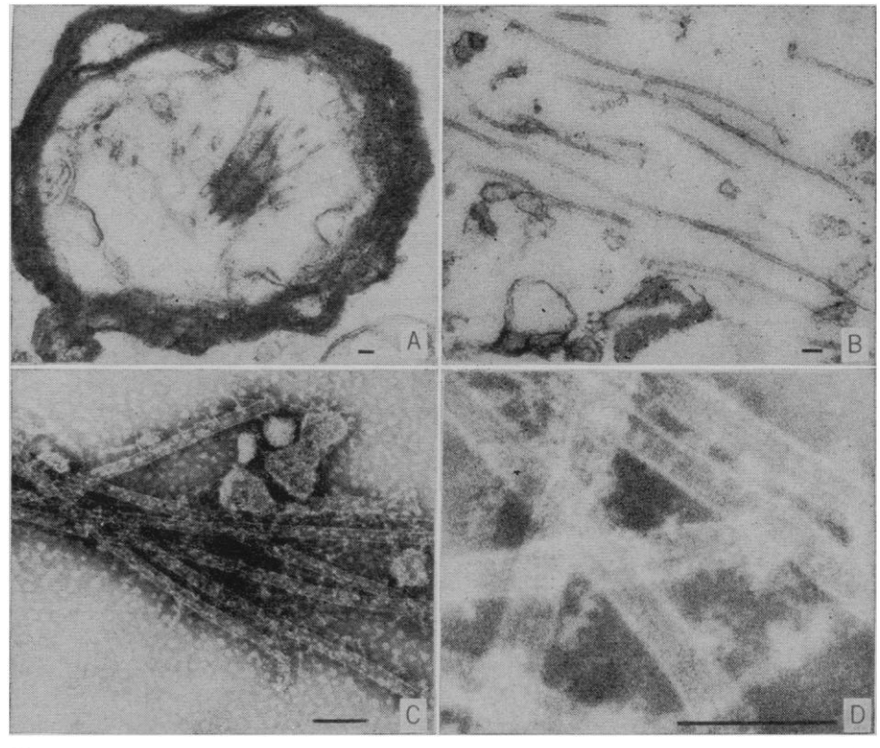

Fig. 1. (A) Partially disrupted myelinated axon from homogenate of rat brain. Microtubules are intact. Plastic-embedded, lead stain (× 20,000). (B) Intact microtubules free in homogenate. Plastic-embedded, lead stain (× 25,000). (C) Microtubules stained negatively with phosphotungstate (× 73,000). (D) Filamentous substructure of microtubules. Negative stain (× 213,000). Each bar represents 1000 Å.