tablished intradermal tumors in guinea pigs caused tumor regression and prevented the development of metastases (*10*). Our result demonstrates the possibility of suppression of primary tumor formation in mice through induction of granulomatous cellular response with a chemically defined substance. Whether the cellular response evoked by cord factor has an immunological basis is under investigation.

A. BEKIERKUNST
I. S. LEVIJ, E. YARKONI
Departments of Medical Bacteriology and Pathology, Hebrew University–Hadassah Medical School, Jerusalem, Israel
E. VILKAS, E. LEDERER
Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France

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Isolation of Filaments from Brain

Abstract. *A method is presented for the isolation of filaments of 90-angstrom diameter from the white matter of bovine brain by first floating the myelinated axons in a centrifugal field and then fractionating the axons on a series of density gradients. This results in a fraction that contains two types of bundles of filaments but few other constituents. The filaments are stable over a wide range of temperatures and at both low and high ionic strength. Their density and their resistance to digestion by ribonuclease and deoxyribonuclease indicate that they are primarily protein. The molecular weight of the subunit is approximately 60,000. The protein does not comigrate with microtubule protein and does not bind cholcicine or nucleotides.*

Neurons and astroglia are both exceptionally rich in intracellular fibrous organelles. Microtubules are present in both types of cells, although far more abundant in neurons, and have been extensively studied from both the morphological and biochemical standpoint (*1*). The neuronal and glial microtubules do not differ significantly from the microtubules of other cells and tissues. Neurofilaments and glial filaments are from 60 to 100 nm in diameter and appear to run in bundles within cells. High-resolution examination with the electron microscope has revealed morphological differences between the two types of filaments, with the glial filament being narrower and being composed of a smaller subunit (*2*).

Davison and his co-workers have characterized a presumptive neurofilament subunit, filarin, which has a molecular weight of 80,000 and which lacks the colchicine- and nucleotide-binding activities of the microtubule subunit (*3*, *4*). In the isolation method of Davison, axoplasm is extruded from the giant axon of the Chilean squid, *Docidicus gigas*, and fractionated to obtain the filaments. The major advantage of this method is the clear neuronal source of the filaments. The disadvantages are that this procedure cannot readily be applied to vertebrate brain, and that the difficulties in keeping a large squid such as *D. gigas* alive in the laboratory rule out many interesting metabolic studies on the neurofilament.

In order to circumvent these difficulties, we have attempted to isolate filaments from mammalian brain. In initial studies, a homogenate of a whole brain was fractionated in discontinuous sucrose density gradients. Fractions that were rich in filaments of 90-Å diameter were found at the interface between 1.5 and 2.0*M* sucrose. However, we could not determine whether these filaments were of neuronal, glial, or mixed origin. Since axoplasm is rich in filaments, the procedure was modified to use as an initial step the new procedure for isolation of axons (*5*, *6*), in which the myelin surrounding the axon functions as a "life vest" to float the axon away from other elements of nervous tissue. Examination with the electron microscope of axons prepared by this method confirmed their suitability as a starting material.

Because the structural integrity of the unmyelinated axons is not important in the filament purification, we have modified the method of DeVries and Norton (*6*) and incorporated it into the isolation scheme.

Myelinated axons were prepared from carefully dissected bovine white matter. White matter (25 g) was homogenized in 625 ml of 0.9*M* sucrose in 0.03*M* phosphate buffer containing 0.01*M* KCl at *p*H 6.5. Homogenzation was either at low speed in a blade-type homogenizer (Sorvall Omni-Mixer, setting 3) or in a glass Dounce homogenizer with a tight pestle. The suspension was then centrifuged in a Beckman L2-65B centrifuge in a Ti-14 rotor at 15,000 rev/min for 15 minutes or in an SW-27 rotor at 10,000 rev/min for 15 minutes. The pads of myelinated axons which had floated to the center or top, depending on the rotor used, were then harvested with care to keep them as free as possible from other materials in the rotor or tube. The pellet and sucrose solution, which consist of cell bodies, nuclei, capillaries, and debris, were discarded.

The pads were rehomogenized in 625 ml of the original homogenizing solution, and the flotation procedure was repeated three to four times. The pads from the final flotation were pooled, washed quickly by resuspension in 200 ml of 30 m*M* phosphate buffer at *p*H 6.5, and pelleted for 10 minutes at 20,000 rev/min. The pads at this stage were composed entirely of myelin and myelinated axons (*6*). The pellet was resuspended in 30 m*M* phosphate at *p*H 6.5 containing $10^{-2}M$ mercaptoethanol, homogenized vigorously in either a blade-type or Dounce homoge-

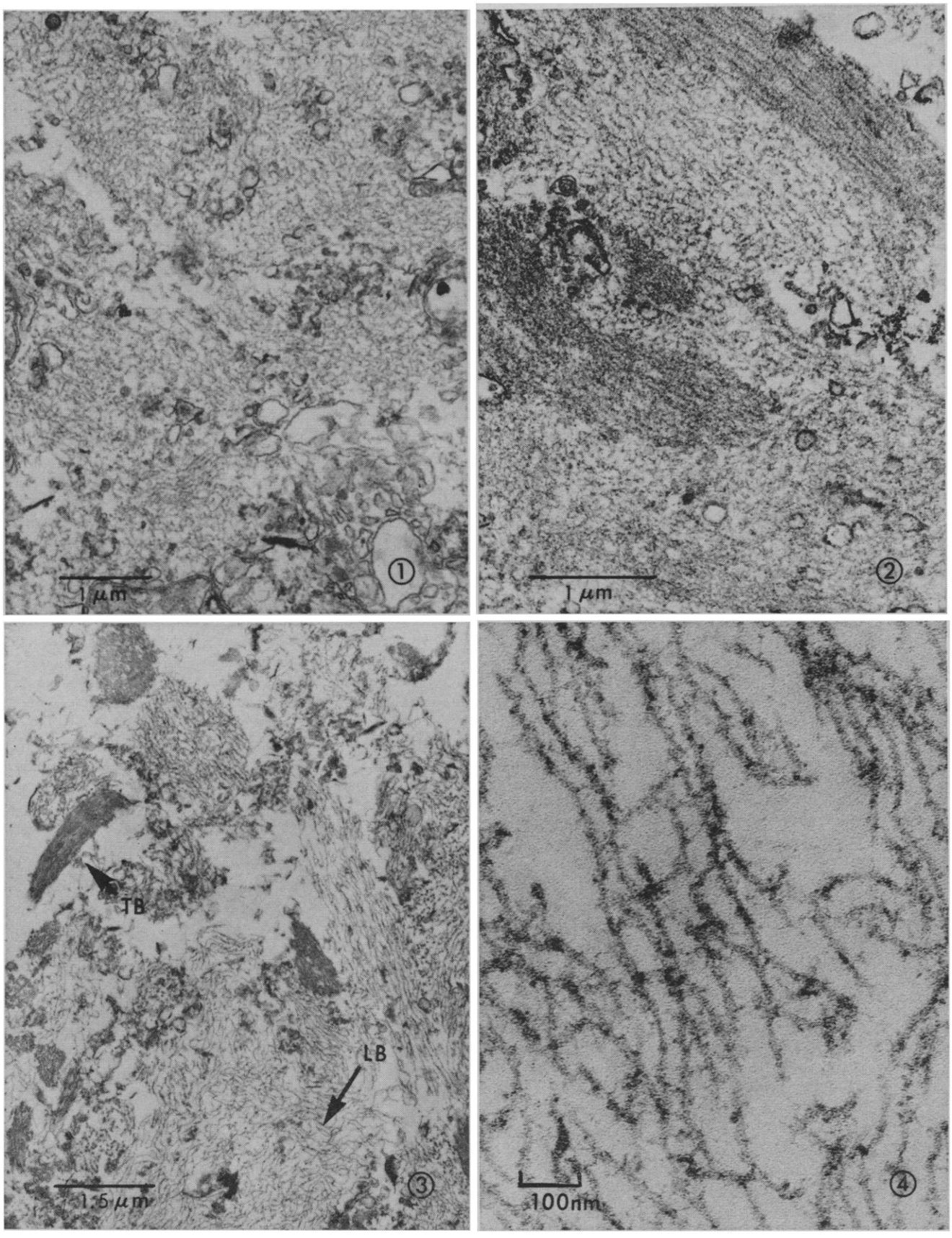

Fig. 1. Crude axoplasmic fraction containing filaments, mitochondria, and membranes (× 17,500). Fig. 2. Fraction from interface between 1.5 and 2.0*M* sucrose on step gradient. Note two types of filament bundles. Membranous contaminants remain (× 25,000). Fig. 3. Filament fraction from CsCl gradient. Two types of filament bundles are present: tight (*TB*) and loose (*LB*) (× 15,000). Fig. 4. Purified filaments. Occasional side arms are visible (× 125,000).

nizer for 1 minute, and allowed to stand in the cold for 1 hour. During this time, the myelin was removed from the axon, and the axoplasmic constituents were released. The solution was made 1.0*M* in sucrose at the end of this period by the addition of crystalline sucrose.

The suspension was centrifuged at 27,000 rev/min (SW-27) or 45,000 rev/min (Ti-14) for 1 hour. The pellets were pooled, and the supernatants and myelin pad were discarded. The resultant material, which we have called crude axoplasm, was composed of membranous vesicles, mitochrondia, and bundles of filaments (Fig. 1). Two distinct types of filament bundles were present. One type was loose and was composed of discrete filaments separated from one another by many filament diameters. The other type of bundle was short and densely staining. In each case the filaments were of roughly 90-Å diameter. All electron microscopy (*7*) was performed on pellets which had been fixed in 2.5 percent glutaraldehyde, postfixed in 1 percent buffered osmic acid, and embedded in Epon.

The crude axoplasm was suspended in the original homogenization medium with a glass homogenizer and centrifuged on discontinuous sucrose density gradients with steps of 1.0, 1.5, and 2.0*M* sucrose in an SW-41 rotor at 41,000 rev/min. All sucrose solutions contained 30 m*M* phosphate and 0.01*M* KCl and were at *p*H 6.5. The interface between 1.5 and 2.0*M* sucrose was rich in filaments (Fig. 2), and a small number of highly purified filaments sediment through the 2.0*M* sucrose step. If Mg^{2+} was added to the preparation at this point, the filaments clumped into a tight mass that was resistant to homogenization. The removal of Mg^{2+} and addition of ethylenediaminetetraacetic acid (EDTA) reversed this effect.

To obtain a greater yield, the material from the interface between 1.5 and 2.0*M* sucrose was sonicated (75 watts, 1 minute, 4°C). Tris(hydroxymethyl)aminomethane maleate (tris maleate) was added to give a concentration of 0.01*M*, CsCl was added to give a concentration of 2.49*M*, and the *p*H was adjusted to 6.8. The suspension was centrifuged for 96 hours at 41,000 rev/min in an SW-41 rotor at 4°C. A sharp band near the top of the tube was composed primarily of membranous material but also contained a few of the tight bundles of filaments. A second band, at a density slightly greater than 1.3 g/cm³, was composed of tight and loose bundles of filaments, with the loose type being about four times more prevalent.

Beneath this band was a strand that appeared fibrous to the naked eye and ran vertically in the center of the tube between the density marker beads at 1.3 and 1.4 g/cm³. The upper half of this strand, when examined ultrastructurally, revealed a composition identical to that of the band immediately above it (Fig. 3). The lower portion of the band had some nuclear contamination. Increasing the duration or intensity of sonication diminished the amount of material in the strand and increased the amount in the band. However, increased sonication also appeared to result in increased losses. The filaments from CsCl gradients were free of nonfilamentous contamination. Numerous side arms were present on the filaments in loose bundles (Fig. 4).

The preparations of purified filaments were diluted, sedimented, and washed in buffer to remove CsCl. The filaments were insoluble at low ionic strength with or without EDTA. They remained intact for at least 24 hours at 37°C and for an indefinite period in the cold. They were not solubilized by exposure to 2*M* KCl, deoxyribonuclease, or ribonuclease, but were readily broken down by trypsin. They were soluble in 8*M* urea, 6*M* guanidine hydrochloride, or 1 percent sodium dodecyl sulfate (SDS). Heating the suspension to 50°C for 15 minutes also appeared to cause a partial solubilization. A determination of sedimentation velocity (*8*) of the protein in 1 percent SDS gave a $s_{20,w}$ value of 2.9.

Colchicine-binding activity is present in the preparations of myelinated axons and in the crude axoplasm, but the amount of colchicine bound per unit of protein falls rapidly with further purification, results suggesting that the filaments lack this activity. Colchicine does not bind to pure filaments. However, the long exposure of these filaments to solutions free from Mg^{2+} makes definitive interpretation of these results difficult. No bound nucleotide was found on the pure filaments, and no binding activity could be demonstrated.

Samples from all stages of the purification procedure were examined by polyacrylamide-gel electrophoresis on the 8*M* urea–SDS system (*9*). Proteins are separated by molecular weight in this system.

In the crude axoplasm a number of bands are present, but the most prominent migrates with a mobility corresponding to a molecular weight of 60,000. Only one major band is present in the preparations of purified filament, and this band comigrates with the major band of the crude axoplasm both in parallel gels and in mixing experiments. A second band with a molecular weight of 120,000 is present in the purified preparations. Densitometry on gels stained with fast green (*10*) reveal this component to be 10 percent of the protein. This probably represents a dimer of the filament subunit, but further work will be necessary to establish this.

Since the molecular weight obtained here is close to that obtained for the microtubule subunits under similar conditions, mixing experiments with tubulins from porcine brain and bovine brain were performed. In all cases the filament protein migrated behind the slower of the two tubulin subunits, which have apparent molecular weights of 53,000 and 56,000 in this gel system (*9*). Since the filament protein has a different molecular weight and does not bind colchicine or nucleotides, a close relationship is unlikely between the filament and tubule proteins, but further studies in which peptide mapping techniques are used will be necessary to settle this.

The major problem with this isolation method is that it yields two morphologically different types of filament bundles. In the final fractions the loose bundles predominate, but a substantial percentage of tight bundles are present. While every effort has been made to avoid contamination with astroglia, one cannot rule out the possibility that one type of bundles, presumably the tighter, is of glial origin. However, we were unable to obtain filaments from gray matter or glioblastoma cultures with this technique. The presence of only one major band in electrophoresis studies also suggests that the filaments are chemically similar and differ only in the amount of cross-linkage.

A second problem is the difference in molecular weight between these filaments and those isolated from the squid axon by Huneeus and Davison (*4*). The lowest value for molecular weight determined on filarin from the squid was over 70,000. The molecular weight of the mammalian filament subunit, determined by similar ultracentrifugal methods, was 58,000 ± 4,000. This might be a species difference. The mam-

malian filament subunit is insoluble in high concentrations of KCl while filarin dissolves readily. The two proteins also differ in solubility in guanidine hydrochloride (*11*). In view of these differences in physical properties, it is hard to draw any conclusions about the homology between squid and mammalian neurofilaments.

The possibility that neurofilaments and neurotubules are polymorphic assembly forms of the same subunits has been raised by morphological studies (*12*). Our data argue against such a direct interconversion. However, the differences in molecular weight between the X subunit of tubulin and the neurofilament are slight and could simply represent differences in glycosylation or other secondary modifications. Therefore, final resolution of these differences must await more detailed studies and peptide maps.

This procedure offers a promising method of isolating neurofilaments from vertebrate brain and for ultimately studying the dynamics of these structures.

MICHAEL L. SHELANSKI*
STEWART ALBERT
GEORGE H. DEVRIES
WILLIAM T. NORTON
Department of Pathology and
Saul R. Korey Department of Neurology, Albert Einstein College of Medicine, Bronx, New York 10461

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* Present address: Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md.

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Ethanol Stimulates Triglyceride Synthesis by the Intestine

Abstract. *In vivo ethanol given acutely or chronically by two dietary means resulted in significant increases in [1-^{14}C]palmitate incorporation into triglyceride by intestinal slices or microsomes derived from intestinal slices. In vitro, 2.6 percent ethanol, an amount comparable to that found in the intestinal lumen of social drinkers, also resulted in significant increases in [1-^{14}C]palmitate incorporation into triglyceride. Pyrazole, an inhibitor of alcohol dehydrogenase, diminished the stimulatory effect of ethanol both in vivo and in vitro. These data may provide a new insight into the effects of alcohol, and specifically on the possible contribution of intestinal triglyceride synthesis to alcoholic hyperlipemia and the alcohol-induced fatty liver.*

Very little research has been done on the effects of ethanol on the intestinal tract in contrast to those on the liver. It has been assumed in the past that most ingested alcohol is absorbed by the stomach, but this appears to be incorrect. Significant amounts of ethanol reach the lumen of the small intestine (*1*).

Work over the past few years indicates that ethanol has a number of effects on intestinal mucosal transport processes. Ethanol has been shown to inhibit the active transport of Na^+ and K^+ and the $(Na^+ + K^+)$ activated adenosine triphosphatases in several tissues and species (*2*). A number of investigators have demonstrated that both in vitro and in vivo administration of ethanol or its presence in the incubation system may lead to interference and inhibition in the uptake and transport of amino acid and glucose (*1*, *3–5*). Israel *et al.* (*1*), studying the effects of ethanol on intestinal L-amino acid absorption in vivo, found in the human small intestine that at a 2 percent concentration of ethanol in the lumen, a concentration that can be found in the human intestine in moderate drinkers, the intestinal absorption of L-methionine was inhibited by 50 percent. Comparable observations have been made by Chang *et al.* (*4*). Recently, Lindenbaum and Lieber (*6*) have presented evidence to indicate that in man alcohol ingestion may lead to a decreased absorption of vitamin B_{12}. Finally, ethanol in vivo has been reported to lead to an interference with fat absorption from the gastrointestinal tract and a retention of a protein meal in the stomach (*7*).

In marked contrast to the inhibitory effects of ethanol on systems cited above, we have observed that ethanol stimulates intestinal cholesterol synthesis (*8*). Mistilis and Ockner (*9*, *10*) recently reported that ethanol introduced into the intestine intraduodenally caused a significant increase in lymph triglyceride content. This report deals with the effect of ethanol on intestinal triglyceride synthesis.

Female Sprague-Dawley rats were fasted 8 hours prior to and 18 hours

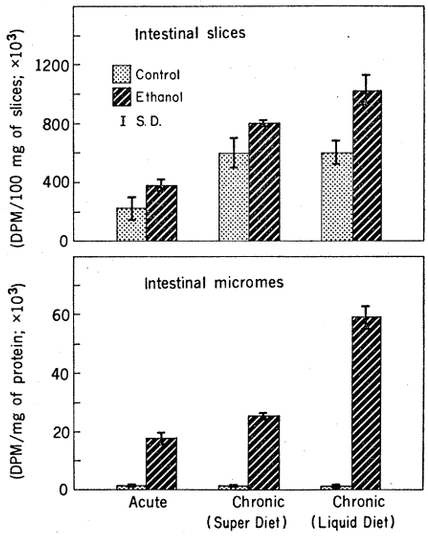

Fig. 1. Effect of ethanol in vivo on the incorporation of [1-^{14}C]palmitate into triglyceride by intestinal slices and microsomes. Intestinal tissue from rats treated with a single acute dose of ethanol (7.5 g/kg), or chronically with ethanol for 28 days with either the high-alcohol liquid diet (*11*) or the super diet (*12*) was excised and prepared as described in the text. Six animals were used in each group. In the case of the intestinal slices, the substrate was 10 μc of [1-^{14}C]palmitate (250 nmole), and incubation was for 1 hour at 37°C. For the microsomal study, the incubation mixture (3.0 ml) consisted of 1.0 ml of microsomal suspension, 25 μmole of D,L-α-glycerophosphate, 10 μmole of adenosine triphosphate, 10 μmole of cysteine (neutral), 20 μmole of potassium phosphate, 10 μmole of $MgCl_2$, 0.15 μmole of coenzyme A, and [1-^{14}C]palmitate (10 μc, 250 nmole) bound to albumin in KCl-tris, *p*H 7.4. Incubations were for 30 minutes at 37°C.