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Identification of Retinoyl Complexes as the Autofluorescent Component of the Neuronal Storage Material in Batten Disease

Abstract. *Cytosomes filled with intensely fluorescent material in the form of curvilinear bodies were isolated by density gradient centrifugation followed by pronase digestion from the cerebral cortex of a child who had died at age 7 from the late infantile form of Batten disease. Forty-three percent of the dry weight of the storage material was extracted by a mixture of chloroform and methanol, leaving a water-insoluble amorphous fluorescent residue. Infrared spectroscopy, proton magnetic resonance spectroscopy, and mass spectrometry of this residue strongly suggested the presence of retinoyl polyenes linked to a small peptide. Base hydrolysis and methanolysis yielded retinoic acid and methyl retinoate, respectively. Ozonolysis yielded a product derived from the substituted cyclohexenyl ring of vitamin A. The results indicate that the fluorescent component of the neuronal storage material is a retinoyl complex and is not derived from peroxidized polyunsaturated fatty acids as previously thought.*

The late infantile type of Batten disease is an inherited neurological disorder of children which starts between 2 and 4 years of age with seizures and visual impairment, followed by progressive intellectual retrogression, blindness, and cerebellar dysfunction, and leads to death between 5 and 9 years. Pathologically, neurons and cells in many other tissues are filled with cytosomes that contain an autofluorescent lipopigment often referred to as ceroid (*1*). By electron microscopy the storage material appears in the form of masses of small crescentic stacks of lamellae referred to as curvilinear bodies (CLB's) (*2*). The term neuronal-ceroid-lipofuscinosis is often used for both the late infantile and the juvenile form of this disease (*3*). The chemical nature of the storage material and biochemical defect in this disease are unknown. A widely held hypothesis is that the fluorophore of both ceroid and lipofuscin is derived from the peroxidation of polyunsaturated fatty acids (*4*). The released malonyldialdehyde could then form Schiff-base complexes with amino groups to form the fluorescent iminopropene chromophores, R−N=CH−CH=CH−NH−R, postulated as the fluorescent products of lipid peroxidation of liver mitochondria and microsomes (*5*). Reports of a *p*-phenylenediamine-dependent myeloperoxidase deficiency in leukocytes in Batten disease (*6*) have not been confirmed by several groups (*7*). We report here chemical studies on isolated cytosomes with curvilinear bodies which identify the fluorescent component as a derivative of retinoic acid.

A 7-year-old child was diagnosed before death as having Batten disease by appendiceal biopsy. Eight hours after death cerebral cortex was dissected free of white matter from the brain of this child. The cerebral tissue, homogenized in a Teflon glass homogenizer in 0.32*M* sucrose, was subfractionated by differential and discontinuous sucrose gradient centrifugation (*8*), and the fractions were monitored for the presence of CLB's by fluorescence and electron microscopy. The bulk of the autofluorescent granules was found in the P_1 or crude nuclear fraction. Vigorous rehomogenization of the P_1 fraction and centrifugation at 53,500*g* for 2 hours on a gradient of 0.8 to 1.8*M* sucrose revealed an intensely fluorescent interface between 1.4 and 1.6*M* sucrose (P_1D fraction), which was found by electron microscopy to be very rich in CLB's. This fraction was diluted with water and sedimented at high speed to give a pellet, which was incubated twice with pronase (*9*). The residue after the pronase digestions was suspended in distilled water and sedimented several times by centrifugation (100,000*g* for 1 hour). The final washed pellet was fixed in glutaraldehyde and osmium tetroxide and embedded. Sections revealed by electron microscopy a fraction consisting almost entirely of CLB's identical in morphology to those seen in intact brain tissue of patients (Fig. 1, A and B). Many repetitions of this procedure yielded 80 mg of the CLB's, which was sufficient for chemical analyses.

Extraction with a mixture of chloroform and methanol (2:1 by volume) removed 43 percent of the dry weight but none of the fluorescent component of CLB's. Standard lipid fractionation procedures (*10*) showed that phospholipids, cholesterol, and free fatty acids were the major lipid classes; neutral glycolipids and gangliosides were not found. The molar ratio of cholesterol to phospholipid was 0.45. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin accounted for 43, 23, 11, and 10 percent, respectively, of the phospholipid phosphorus, with small amounts of phosphatidylinositol and lysolectithin and 6 percent unidentified. The major fatty acids of the total phospholipids were palmitic, stearic, and oleic acids, with 10 percent arachidonic and 6 percent docosahexenoic acids. However, electron microscopy of the fluorescent lipid-free residue revealed only an electron-opaque amorphous material, with complete loss of the lamellated curvilinear profiles.

The fluorescent component of lipid-free CLB's was not solubilized by 8*M* urea, 10 m*M* EDTA, or any common organic solvent except a mixture of dimethyl sulfoxide and water (2:1 by volume). The fluorescence emission maximum in the latter solvent system was at 425 to 430 nm, excited at 355 to 360 nm. Base hydrolysis (4*N* NaOH for 12 hours at 100°C) solubilized the CLB's without destroying the fluorescence, part of which could be extracted with dichloromethane. Acidification of the dichloromethane quenched the fluorescence. Ultraviolet spectra of the unhydrolyzed CLB's in dimethyl sulfoxide–water showed a maximum at 280 nm with a broad shoulder extending to 340 nm. After base hydrolysis the 280-nm peak was not present, but in ethanol a peak between 340 and 350 nm became apparent. Infrared spectra of the lipid-free CLB's in KBr pellets were informative. A broad peak at 3300 to 3400 cm^{-1} was assigned to N–H and O–H stretching, small absorptions at 2860 to 2960 cm^{-1} to C–H stretchings of CH_3- and CH_2- groups, strong peaks at 1650 and 1520 cm^{-1} to amide I (C=O stretching) and amide II (N–H bending), and a small peak at 1380 cm^{-1} to $-CH_3$ bending. Deuterium exchange shifted the amide II peak from 1520 to 1400 cm^{-1} and revealed a small shoulder at 1600 cm^{-1} due to C=C stretching. The 1650-

cm^{-1} peak disappeared on base hydrolysis, and thus amide and N=C groups were not part of the fluorophore. Fourier transform proton magnetic resonance spectroscopy of the dichloromethane extract from the base-hydrolyzed CLB's yielded the results shown in Table 1. The absorptions indicated the presence of a polyene-type structure with branched methyl groups. Nuclear magnetic resonance spectra of unhydrolyzed, lipid-free CLB's dissolved at 100°C in d_6-dimethyl sulfoxide with a trace of D_2O gave all the absorptions listed in Table 1 but the relative intensities of the peaks were different because of peptide contributions. At this stage of the chemical studies, we thought that intact CLB's consisted of a polyamide polymer that contained short unsaturated aliphatic sequences, but we were unable to explain the clear indications of saturated methyl groups and branched methyl groups attached to unsaturated carbon atoms.

Direct-inlet mass spectrometry (*11*) of the lipid-free CLB's at probe temperatures between 50° and 80°C gave spectra with fragment ions at a mass-to-charge ratio (*m/e*) of 255 and below identical to those reported in retinol, retinoic acid, and methyl retinoate (*12*). The spectra were characteristic of the fragmentation pattern of a polyene. The ion of *m/e* 255 is due to cleavage α to the C-15 of retinyl compounds and represented 1 percent of the total ion current. Prominent ions of *m/e* 213, 185, 173, 159, 145, 133, 121, 119, 107, 105, 95, 93, 91, 81, 69, 55, 43, and 41 were present in the CLB spectra and are typical of the hydrocarbon breakdown of retinyl compounds (*12*). The mass spectral evidence for retinyl-type compounds was consistent with the results from magnetic resonance, infrared, and fluorescence spectra.

Further evidence for the retinyl character of the fluorescent component in CLB's was obtained by ozonolysis studies. The acid hydrolysate (1*N* HCl at 100°C for 10 hours) from CLB's was passed through Sephadex G-10 (*13*) to remove amino acids. The void volume of the Sephadex column (fluorescent) was treated with methanol-HCl, the acid removed, and the residue dissolved in methanol–ethyl acetate (1:1 by volume) and subjected to ozonolysis at −60°C for 2 minutes. Fluorescence was immediately destroyed. The ozonolysis products were reduced with sodium borohydride, acetylated, and subjected to gas chromatography–mass spectrometry (*14*). The mass spectrum of the major product was identical to that obtained when β-carotene was subjected to the same sequence of reactions. The ozonolysis product was most probably derived from cleavage of the C–5,6 double bond of the cyclohexenyl ring and the C–7,8 double bond of the polyene side chain of retinyl compounds.

Table 1. Lipid-free CLB's (5 mg) were hydrolyzed with base and the hydrolysate extracted several times with dichloromethane. The solvent was removed and the residue dissolved in $CDCl_3$. Nuclear magnetic resonance spectra were recorded in a Bruker HFX-10 with a Fourier transform mode at 90 Mhz and 300°K with 2500 scans. The signal lock was on the deuterium nucleus. Chemical shifts are parts per million from tetramethylsilane.

Chemical shift (ppm)	Approximate number of protons	Assignment
0.92 (multiplet)	6	$>C<^{CH_3}_{CH_3}$
1.28	4	CH_2–CH_2
1.62	8–9	CH_3–C=C
2.05	2	CH_2–C=C
3.62	<1	CH_2–OH
5.15, 5.32	2–3	*cis*, *trans*-C=CH

After we realized that vitamin A derivatives appeared to be involved as the fluorescent compound of CLB's in a complex with peptide, we exercised much more care in the hydrolysis conditions. Treatment of CLB's with 0.5*N* KOH in 50 percent aqueous methanol at 60°C for 30 minutes yielded, after acidification, a small amount of fluorescent material extractable in hexane. Thin-layer chromatography on silica gel G in ethyl acetate showed a fluorescent spot comparable in mobility to all-*trans* retinoic acid, which gave a transient blue color with antimony trichloride stain. This hydrolysis was mild and therefore incomplete. The residue was treated with 3 percent methanol-HCl at 70°C for 90 minutes. The methanolysis products were evaporated to dryness and extracted with hexane. Silica gel thin-layer chromatography in benzene gave a spot similar in mobility to all-*trans* methyl retinoate. Similarly, gas chromatography (on Silar 10-C at 180°C) of the methanol-treated CLB's and methyl retinoate showed similar retention times, but some degradation had occurred for both. Mass spectra of the gas chromatographic peaks were identical but differed from the published spectra of methyl retinoate obtained by direct-inlet mass spectrometry at low temperature (*12*). Retinyl compounds are notoriously unstable at high temperatures. Amino acid analysis (Beckman Autoanalyser) of acid-hydrolyzed CLB's showed the presence of aspartic and glutamic acids, serine, threonine, glycine, alanine, isoleucine, and leucine. The evidence indicates that the retinoic acid is complexed, possibly in amide linkage to a small peptide.

The identification of retinoyl complexes that account for more than 50 percent of the dry weight of the storage material in neurons from Batten disease opens up an entirely new approach to elucidation of the biochemical pathogenetic mechanism in this condition. Retinoic acid is known to be essential for growth and differentiation of tissues and has

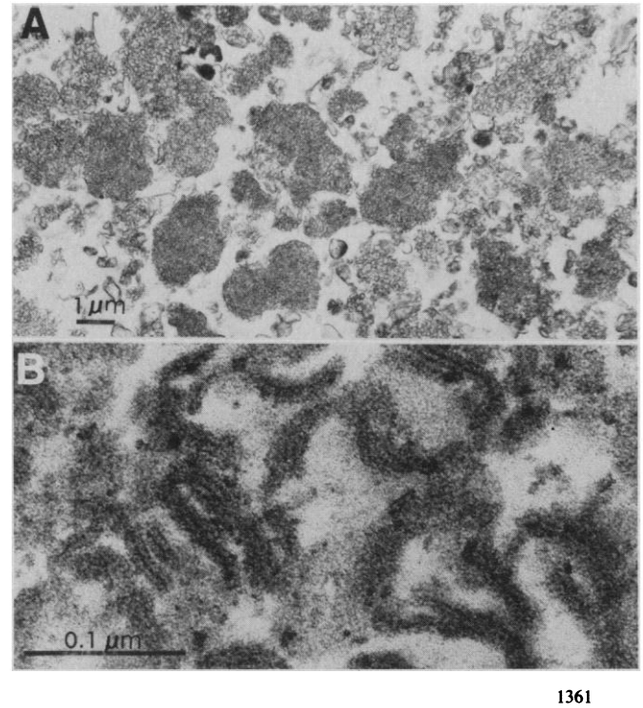

Fig. 1. (A) Low-power electron micrograph of pronase-treated P_1D fraction, showing a high concentration of characteristic curvilinear bodies. (B) High magnification demonstrates that the lamellation of the curvilinear profiles is preserved in the P_1D fraction.

functions separate from those of retinol or retinal (*15*). Whether retinoic acid or a tissue metabolite derived from it is the biologically active compound is unclear (*15*). Retinoic acid, to our knowledge, has never been reported as present in brain. Nevertheless, a distinct possibility is that the gene defect in Batten disease involves an enzyme or enzymes that catabolize retinoic acid. It is of interest that the lamellated character of CLB's requires the presence of cholesterol and phospholipids and that vitamin A derivatives can readily form lamellated micelles with phospholipids (*16*). Finally, since lipofuscin or the so-called age pigments in neurons have fluorescent properties similar to those of CLB's, it seems important to reinvestigate their chemical composition in the light of this research (*17*).

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Nitrogen Fixation in Grass-*Spirillum* Systems

Smith *et al.* (*1*) indicated that nitrogen fixation by *Spirillum lipoferum* reduced the fertilizer requirement for two grasses by up to 0.6 kg of nitrogen per hectare per day. The number of bacteria applied was not exactly specified, but assuming rows 18 cm apart, about 4×10^{12} bacteria would have been applied per hectare. Each applied bacterium thus seems to be responsible for replacing (but not necessarily fixing) on the order of 1.4×10^{-10} g of nitrogen per day, a rather astounding feat for an organism which must at the onset weigh 1/10 to 1/100 of that. Since the growth rate of *S. lipoferum* and its efficiency of root infection are unknown, it is impossible to estimate the true efficiency of nitrogen fixation. Nonetheless, the association seems to be very efficient indeed. Smith *et al.* do not address this point and do not report complete nitrogen balances. Moreover, they do not begin to satisfy the spirit of Koch's postulates of microbial causality by correlating nitrogenase activity with growth enhancement in the putatively infected plants. In the light of the recent demonstration by Brown (*2*) that *Azotobacter paspali* may enhance grass growth by producing growth regulating substances, it seems to me premature to conclude that it is *S. lipoferum*'s ability to fix nitrogen which is enhancing grass growth.

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Carefully controlled experiments have demonstrated that the bacteria used in our experiments can invade grass root tissue (*1*) and that the colonies that result can reduce acetylene to ethylene (*2*). Further, Burris *et al.* (*3*) have demonstrated Koch's postulates working with monoaxenic cultures. These observations clearly justify testing under field conditions to determine whether inoculation may induce nitrogen fixation. We did not suggest that the cells applied to the soil, to achieve inoculation, could fix a significant amount of nitrogen. We assumed that a dynamic population increase would occur, as is well known with *Rhizobium* systems.

The report by Brown is not the first to demonstrate production of plant growth substances by bacteria. Indeed, many bacteria are known to produce such substances. However, this has not been shown with *Spirillum lipoferum*, whereas the ability to fix nitrogen has. We agree that the data at hand are limited and do not show unequivocally the reason for enhanced plant growth. We hope that other scientists will be encouraged to contribute to the solutions of problems confronting a nitrogen-deficient world.

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