Ontogenetic Study of a Myelin-Derived Fraction with 2':3'-Cyclic Nucleotide 3'-Phosphohydrolase Activity Higher than that of Myelin

By THOMAS V. WAEHNELDT

Max-Planck-Institut für Experimentelle Medizin, Forschungsstelle Neurochemie,
3400 Göttingen, West Germany

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A membrane fraction (SN 4), prepared from developing and mature rat forebrain by hypo-osmotic treatment of microsome (microsomal-fraction)-free myelin, appears to be closely related to the myelin-like fraction. Fraction SN 4 shows 2':3'-cyclic nucleotide 3'-phosphohydrolase activity higher than that of the parent myelin. Electrophoresis reveals a multitude of bands with the Wolfgram protein as the main component.

Morphological observations (Peters *et al.*, 1970; Peters & Vaughn, 1970) indicate that the ensheathing of axons occurs as a continuous process in which initial formation of occasional loose lamellae and final multilayered myelin compaction can be regarded as developmental extremes. Attempts to understand the biochemical basis of compact myelin formation have lead to the isolation and characterization of immature material from developing brain, such as early myelin and myelin-like material (Banik & Davison, 1969; Agrawal *et al.*, 1970, 1973, 1974; Davison, 1971).

In a previous communication the isolation of a microsome (microsomal-fraction)-free membrane fraction (SN 4) was described (Waehneltdt & Mandel, 1972). This fraction consisted mostly of membrane fragments, and the Wolfgram protein (Wolfgram, 1966) was the major component. Although fraction SN 4 was isolated from mature rat brain it was suggested (Waehneltdt & Mandel, 1972) that this fraction is related to the myelin-like material (Agrawal *et al.*, 1970) of young rapidly developing animals. This is substantiated by the present study, in which the isolation of fraction SN 4 is extended to immature animals. It is also shown that fraction SN 4 displays higher 2':3'-cyclic nucleotide 3'-phosphohydrolase specific activity than does the multilayered myelin and much higher activity than that of the myelin-like fraction (Agrawal *et al.*, 1973).

Wistar-strain rats (TNO/W 70) were used. Age of animals and numbers of brains in duplicate experiments are indicated in Table 1. Animals labelled adult were older than 6 months. Fraction SN 4 and myelin were isolated as described by Waehneltdt & Mandel (1972), with the following alterations. (1) A 5% (w/v) total homogenate of rat forebrain (Waehneltdt & Neuhoff, 1974) was prepared. (2) Isolation of a nuclear pellet was omitted. (3) The myelin-containing pellet was washed three times at 11000*g*_{av.} (4) No further purification of myelin on 0.80M- and 0.70M-

sucrose was carried out. Electrophoretic fractionation in sodium dodecyl sulphate-polyacrylamide gels was performed as described by Waehneltdt & Neuhoff (1974). Staining was done with Coomassie Blue in methanol-acetic acid (Agrawal *et al.*, 1972). A gel scanner (Zeiss, ZK 4) with attached strip recorder (Servogor S, RE 544) was used for monitoring and integrating the extinction output. Acetylcholinesterase (EC 3.1.1.7) and 2':3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16) activities were assayed by the methods of Ellman *et al.* (1961) and Prohaska *et al.* (1973) respectively. Crystalline bovine serum albumin served as a protein standard in the protein-determination procedure of Lowry *et al.* (1951).

During ontogeny myelin proteins are rapidly accumulating from 0.19mg of protein/g of forebrain of 15-day-old animals to 7.6mg of protein/g of forebrain of adult animals. These data are consistent with previous observations (Waehneltdt & Neuhoff, 1974; Zgorzalewicz *et al.*, 1974). Fraction SN 4, by contrast, contains only 0.045mg of protein/g of forebrain of 15-day-old animals and gradually increases to 0.5mg of protein/g of forebrain of adult animals, implying that only small amounts of membrane fragments are found in the supernatant of hypo-osmotically shocked microsome-free myelin after differential centrifugation. The ratio myelin/fraction SN 4 increases from about 4 in 15-day-old animals to about 15 in adult animals, indicating the presence of relatively large amounts of osmotically labile membranes attached to rather little compacted myelin in young rats. The situation is reversed in older animals, with their abundance of multilayered layered and osmotically stable, i.e. cytoplasm-free, myelin.

The activities found for cyclic nucleotide 3'-phosphohydrolase (Table 1), an enzyme not only localized in compact myelin but also regarded as closely related to the process of myelination (Poduslo, 1975), are substantially higher in fraction SN 4

Table 1. *Distribution of 2':3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16) activity in total homogenate and in myelin subfractions from rat forebrain of different ages*

Fractions were prepared as described in the text. Enzymic activities are expressed as μmol of 2':3'-cyclic AMP cleaved/h per mg of protein. *F*, numbers of forebrains used in two experimental series. Triplicate analyses were performed for each series and their means are shown. TH, total homogenate; MY, myelin; SN 4, fraction derived from myelin by osmotic shock.

Age of animals (days)	<i>F</i>	Mean enzymic activity in subcellular fraction		
		TH	MY	SN 4
15	16, 16	47	1284	1847
18	16, 14	66	1306	2048
21	16, 12	115	1559	2682
30	10, 10	232	1616	3535
Adult	7, 7	299	1210	3272

than in myelin at all ages examined. No such high values (approx. 11 times that of the total homogenate) have yet been described for an untreated membrane fraction; still higher cyclic nucleotide 3'-phosphohydrolase activities have been reported, however, for solubilized bovine brain cyclic nucleotide 3'-phosphohydrolase (Guha & Moore, 1975). The values for the total homogenate and for myelin agree with those of Agrawal *et al.* (1973, 1974), particularly when it is considered that these authors examined total brain from 15-day-old animals, which represents a later stage in overall myelination (Zgorzalewicz *et al.*, 1974), as compared with the forebrain used in the experiments described in the present paper. It was observed that fraction SN 4 of forebrain from 15-day-old animals exhibits more than twice the cyclic nucleotide 3'-phosphohydrolase activity of the myelin-like fraction of total brain from 15-day-old animals (Agrawal *et al.*, 1973). Subfractionation by differential centrifugation of fraction SN 4 from adult rats yields slowly sedimenting fractions with cyclic nucleotide 3'-phosphohydrolase activities higher than $4000 \mu\text{mol}$ of 2':3'-cyclic AMP cleaved/h per mg of protein (T. V. Waehneltdt, unpublished work). Acetylcholinesterase activities in preparations of fraction SN 4 from forebrain of 15-day-old animals were lower ($5.04 \pm 0.29 \mu\text{mol}$ of acetylthiocholine hydrolysed/h per mg of protein) than in the myelin-like fraction (8.25 ± 0.13) (Agrawal *et al.*, 1973) from total brain of 15-day-old animals.

Electrophoretic analyses on sodium dodecyl sulphate-polyacrylamide gels of fraction SN 4 and myelin fraction illustrate close similarities between the banding patterns with large differences in staining intensity of individual proteins (Plate 1). The major myelin proteins, i.e. proteolipid protein,

DM-20 protein (Agrawal *et al.*, 1972), plus large and small basic proteins, are predominant in myelin fraction (averaging 75% of all proteins); however, they are substantially decreased in the SN 4 fractions (averaging 24%). The reverse is valid for the Wolfgram protein, which is the major protein component in SN 4 fractions (averaging 27% compared with 8% in the myelin fraction), with values up to 50% in SN 4 subfractions from adult rats. In addition, a periodate-Schiff-positive high-molecular-weight protein is found in substantial amounts in fraction SN 4 (averaging 11%), in contrast with the myelin fraction. This component is probably the myelin-associated glycoprotein described by Quarles *et al.* (1972).

During the preparation of fraction SN 4 care was taken to avoid microsomal contamination by three-fold washing of the crude homogenate with iso-osmotic medium. Although only very small amounts of compact myelin were found during the initial phases of myelination, this very compaction facilitates the isolation of adhering osmotically and mechanically labile membranes. Compared with the myelin-like material (Agrawal *et al.*, 1973, 1974), the resulting fraction SN 4 is very high in cyclic nucleotide 3'-phosphohydrolase activity and lower in acetylcholinesterase activity. The cyclic nucleotide 3'-phosphohydrolase activity is regarded as the only positive evidence linking Agrawal's myelin-like material to myelin (Agrawal *et al.*, 1974). Since fraction SN 4 studied here has even higher cyclic nucleotide 3'-phosphohydrolase activity than myelin, a connexion between fraction SN 4 and the myelin-like material is strongly implicated. In this sense fraction SN 4 constitutes a purified myelin-like fraction, with cyclic nucleotide 3'-phosphohydrolase as a positive marker for the myelin precursor membrane paralleled by high Wolfgram protein content.

Although fraction SN 4 is operationally derived from compact myelin through osmotic shock, it may represent a stage in the membranous continuum ranging from the oligodendroglial plasma membrane to multilamellar myelin (Agrawal *et al.*, 1974; Poduslo, 1975; Sabri *et al.*, 1975). Among others, the Wolfgram protein (Waehneltdt & Neuhoﬀ, 1974), as well as the 2':3'-cyclic nucleotide 3'-phosphohydrolase (Agrawal *et al.*, 1974) and the myelin-associated glycoprotein (Matthieu *et al.*, 1973; Poduslo *et al.*, 1975), are possibly intrinsic components of this transitory stage that decrease through gradual insertion of major myelin proteins and other compounds during the formation of mature myelin.

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EXPLANATION OF PLATE I

Electrophoresis of sodium dodecyl sulphate-extracted proteins from myelin subfractions of different ages

Experimental details are given in the text. (a) SN 4, fraction derived from myelin by osmotic shock; (b) MY, myelin; SBP, small basic protein; LBP, large basic protein; DM-20, myelin protein described by Agrawal *et al.* (1972); PLP, proteolipid protein; WP, Wolfgram protein; GP, periodate-Schiff-positive protein.

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