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Protein Determinants of Myelination in Different Regions of Developing Rat Central Nervous System

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Measurements of several different protein determinants correlated with the time and rate of myelination in five areas of the central nervous system are presented. The deposition of protein in the subcellular fraction corresponding to the density of adult myelin, the appearance of basic protein characteristic of myelin, the change in proportions of the individual myelin proteins, the appearance and distribution of the myelin marker 2′:3′-cyclic nucleotide 3′-phosphohydrolase, and the results of morphological studies of purified myelin are compared. According to these various criteria, and in agreement with the morphological observations of others, myelin appears earliest in the spinal cord, then in the brain stem, and latest in the cerebral hemispheres. Multilamellar myelin was observed in the rat brain stem and spinal cord as early as 5 days of age. The relative proportion of the individual myelin proteins changed with myelin maturation in all areas, with the larger basic protein decreasing reciprocally with increase of the smaller basic protein. The proportion of Wolfgram protein also decreased with maturation. Larger proportions of the enzyme 2′:3′-cyclic nucleotide 3′-phosphohydrolase were located in the microsomal fraction at early ages. During development the enzyme activity gradually became associated more with a fraction of a density corresponding to adult myelin, suggesting the presence of precursor membrane fragments in microsomal fractions in the early stages of myelination before compact myelin formation. A significant proportion of the total nucleotide phosphohydrolase activity of the homogenate could not be recovered in subcellular fraction at early ages, but the recoveries of the enzyme increased with maturation and the activity was found more in the myelin fraction.

Histological examination of the developing central nervous system shows that the onset of myelin deposition occurs at different times in different regions of the central nervous system. Generally the phylogenetically older parts of the nervous system, irrespective of species, form myelin earlier than the anterior areas. Thus myelin appears in the spinal cord first, then in the brain stem, cerebellum and forebrain. During development, biochemical changes in protein and lipid composition have been observed in myelin isolated from whole brain (Horrocks *et al.*, 1965; Davison *et al.*, 1966; Cuzner & Davison, 1968; Eng & Noble, 1968; Eng *et al.*, 1968; Banik & Davison, 1969; Dalal & Einstein, 1969; Savolainen *et al.*, 1972; Matthieu *et al.*, 1973; Norton & Poduslo, 1973; Banik *et al.*, 1974; Fishman *et al.*, 1975). Fewer reports, however, have dealt with the composition of myelin from different regions of adult and developing brain (Mehl & Wolfgram, 1969; Morell *et al.*, 1973; Smith, 1973; Lees & Paxman, 1974; Zgorzalewicz *et al.*, 1974). In one study the alteration in the lipid composition and metabolism of myelin isolated from different areas of the developing rat brain (Smith, 1973) has been correlated with the temporal pattern of the appearance and maturation of myelin as observed by morphological studies (Jacobson, 1963). In the present study we have attempted to extend biochemical correlations of myelin appearance in the different areas to the appearance of myelin-specific proteins and a myelin-specific enzyme, 2′:3′-cyclic nucleotide 3′-phosphohydrolase. Although this enzyme has been used as an index of myelination, the present study shows that the activity may be present in a subcellular fraction of a different density early in development, and then appears to be transferred to the fraction with the characteristic density of the myelin membrane. A preliminary report of this work has appeared elsewhere (Banik & Smith, 1975).

Materials and Methods

Animals

Wistar rats of either sex were used throughout these experiments. The rats were decapitated and the brains

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and spinal cords quickly removed. The different brain regions were dissected out as previously described (Smith, 1973), and placed in beakers in an ice bath until weighed and homogenized. At least 10–15 brains were pooled for experiments on animals of ages between 3 and 15 days old, five brains were pooled for the 25-day-old rats, and two or three brains were used for each point representing older animals.

Preparation of tissues

Brain and spinal cord specimens were homogenized in 0.32M-sucrose in a Teflon/glass homogenizer to make a 5% (w/v) homogenate. Purified myelin was isolated by the method of Smith (1968). The homogenate was centrifuged for 45min at 10000*g* and the supernatant kept for microsomal preparation. The pellet was homogenized in 20ml of 0.88M-sucrose and 10ml of 0.32M-sucrose was layered over the 0.88M-sucrose suspension. The contents were centrifuged at 75000*g* for 50min. The crude myelin that accumulated at the interface was removed, diluted threefold with cold water, and centrifuged at 75000*g* for 15min. The pellet was washed with 3×30ml of water, and pelleted after each wash at 75000*g* for 15min. The washed myelin pellet was again homogenized in 20ml of 0.88M-sucrose, 10ml of 0.32M-sucrose was layered on top and the discontinuous gradient centrifuged as before. The myelin layer at the interface was collected and washed three times with water as above. The microsomal fractions were prepared from the initial 10000*g* supernatant fraction by centrifugation at 78000*g* for 1h in a Spinco model L centrifuge.

Enzyme assay

Acetylcholinesterase (EC 3.1.1.7) was determined by the method of Ellman *et al.* (1961) and ATPase* (EC 3.6.1.3) was measured as described by Schwartz *et al.* (1962). Nucleoside 2′:3′-cyclic monophosphate 3′-phosphodiesterase (EC 3.1.4.16) or adenosine 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity was measured by the method of Banik & Davison (1969). The tissue or cell fraction was prepared for this assay by treatment with an equal volume of 1.0% Triton X-100 in ice for 60min with occasional stirring as previously described (Agrawal *et al.*, 1970). Adenosine 2′:3′-cyclic phosphate (sodium salt), acetylthiocholine iodide, 5,5′-dithiobis-(2-nitrobenzoic acid), and ATP (disodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Protein separation

Total protein was determined by the method of Lowry *et al.* (1951) with Versatol (General Diagnostics, Morris Plains, NJ, U.S.A.) as standard. Proteins were separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Banik *et al.* (1974). Gels were stained overnight with Coomassie Blue, destained, and densitometric scans of the gels were made at 560nm with a Gilford model 250 spectrophotometer equipped with a linear transport system and strip chart recorder. Protein peaks were quantified by triangulation of the peaks.

* Abbreviation: ATPase, adenosine triphosphatase.

Results

Morphology

Examination by electron microscopy of the fraction collecting between 0.32M- and 0.88M-sucrose from the 5-day-old animals revealed the appearance of compact myelin in those from the spinal cord and brain stem only. Generally the numbers of lamellae in compact myelin from brain stem was less than in myelin from spinal cord. Many structures, which appeared to consist of single lamellae and often formed vesicles, were also observed. This fraction isolated from the cerebral hemisphere contained only amorphous lamellar structures with no compaction of membranes (Plates 1*a*, 1*b* and 1*c*). These structures could possibly have represented an early stage of myelination where the axon is enveloped by the cell process. Electron-microscopic examination of the microsomal fractions (Plates 2*a*, 2*b* and 2*c*) obtained from different areas of 5-day-old rat central nervous system revealed numerous vesicular membranous structures, some of which were larger than microsomal membranes.

Assay of membrane marker enzyme

Acetylcholinesterase and Na^+, K^+, Mg^{2+}-activated ATPase, marker enzymes for microsomal particles, were used to ascertain the extent of contamination of myelin fractions and characterization of microsomal fractions (Table 1). Recoveries of acetylcholinesterase in total myelin and microsomal fractions of animals 5, 15 and 40 days old were 0.21–3.8 and 12.5–34.1% respectively of the activity in the total homogenate. Recoveries of ATPase were 0.23–1.14% in myelin and 12.4–20.6% in the microsomal fraction.

Myelin protein content during development in different regions

The rate of accumulation of total myelin protein, defined as protein in that material collecting at the interface between 0.32M- and 0.88M-sucrose after centrifugation, differed in the various regions studied. The most significant increase in the amount of myelin protein was found at about 15 days in spinal cord and brain stem, reflecting the high proportion of white matter in these structures, whereas the rapid phase of myelin protein accumulation did not begin until after 15 days in the thalamic area, cerebellum and cerebral hemisphere (Fig. 1).

Table 1. *Acetylcholinesterase and ATPase activity in purified myelin and microsomal fractions isolated from different regions of developing rat central nervous system*

Methods of assaying enzyme activities are given in the Material and Method section in the text. Abbreviations: WH, whole homogenate; CH, cerebral hemisphere; Thal., thalamus; SC, spinal cord.

Age (days) ...	5			15			40		
Regions ...	CH	Thal.	SC	CH	Thal.	SC	CH	Thal.	SC
Acetylcholinesterase									
WH activity (μmol of substrate hydrolysed/h per g wet wt. of tissue)	144	300	390	354	420	582	912	630	546
Myelin activity (μmol of substrate hydrolysed/h per g wet wt. of tissue)	0.30	1.74	2.61	3.40	2.40	22	8.43	2.50	3.60
Microsomal activity (μmol of substrate hydrolysed/h per g wet wt. of tissue)	29.81	61.35	66.04	80.09	94.56	148.32	114.06	150.02	102.61
% of total WH activity recovered in myelin	0.21	0.58	0.67	0.96	0.57	3.8	0.92	0.40	0.66
% of total WH activity recovered in microsomal fraction	20.71	20.30	16.93	23.04	18.54	34.17	12.52	23.85	18.73
Myelin specific activity (μmol/h per mg of protein)	3.10	6.00	4.92	5.10	3.46	4.40	0.32	0.22	0.20
Microsomal specific activity (μmol/h per mg of protein)	4.05	7.60	9.60	11.32	15.83	22.40	15.62	14.11	12.05
Total ATPase									
WH activity (μmol of P_i released/h per g wet wt. of tissue)	372	851	613	1010	1265	1392	2268	1958	1769
Myelin activity (μmol of P_i released/h per g wet wt. of tissue)	0.34	1.80	1.41	6.23	9.10	12.05	17.11	23.02	16.19
Microsomal activity (μmol of P_i released/h per g wet wt. of tissue)	77	141	118	140	171	187	289	326	273
% of total WH activity recovered in myelin	0.92	0.38	0.23	0.61	0.72	0.86	0.75	1.14	0.90
% of total WH activity recovered in microsomal fraction	20.62	16.65	19.39	13.82	13.61	13.70	12.42	16.70	15.41
Myelin specific activity (μmol/h per mg of protein)	3.40	5.60	2.14	5.91	6.70	2.43	2.41	2.08	0.88
Microsomal specific activity (μmol/h per mg of protein)	10.30	17.40	17.24	19.90	34.21	41.53	39.61	30.70	32.20

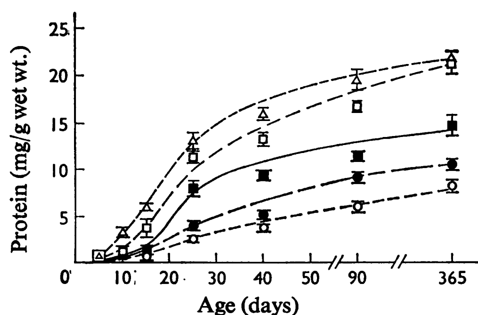

Fig. 1. *Total protein content in subcellular fractions corresponding to the density of adult myelin isolated from different regions of the rat central nervous system during development*

Protein was determined by the method described in the text. Bars represent S.D. of four experiments; ●, cerebellum; ○, cerebral hemisphere; □, brain stem; ■, thalamic area; △, spinal cord.

Changes in composition of myelin protein

The total myelin protein was considered to be the sum of the two basic proteins, the proteolipid protein and the high-molecular-weight protein corresponding to the protein described by Wolfgram (1966) as shown on the gel scan on Plate 3. The basic encephalitogenic protein was detectable in the myelin samples isolated from spinal cord and brain stem from 3- and 5-day-old rats, but not in cerebral hemisphere (Plates 4*a* and 4*b*). At 10 days, basic protein was demonstrable in myelin from all regions studied.

The protein pattern of microsomal fractions when separated on polyacrylamide gels also showed some changes with maturation. The microsomal fraction isolated from various parts of the central nervous system during early stages of development contained, in addition to high-molecular-weight proteins, a

protein band corresponding to myelin basic protein (Plate 5).

The relative proportions of basic proteins changed during development with the pattern of change similar in all areas (Figs. 2*a* and 2*b*). During early stages of myelination the amount of higher-molecular-weight basic protein present as estimated by Coomassie Blue binding was greater than that of the smaller basic protein, but with increasing age the relative amounts of the larger basic protein decreased, whereas the smaller gradually increased in all regions.

Except in the brain stem, a dramatic decrease in the proportion of Wolfgram protein was observed with development, with this protein comprising 25–30% of the total at 10 days after birth, decreasing to 10% or less at 40 days. In general, the stable adult pattern was attained by 40 days of age and did not change thereafter in any area. The DM-20 protein (Agrawal *et al.*, 1972) was not detected in these gels under the conditions in which the proteins were separated.

Changes in 2′:3′-cyclic nucleotide 3′-phosphohydrolase during development

The total activity of the myelin marker enzyme 2′:3′-cyclic nucleotide 3′-phosphohydrolase increased significantly during development in all the different regions of the brain (Tables 2 and 3). The activity per g wet wt. of original tissue in the tissue homogenate of each area gradually increased to reach a maximum activity at 25 days in the spinal cord, brain stem, cerebellum and thalamus, and at 90 days in the cerebral cortex (Table 3). The timing of appearance of this enzyme differed, however, in the different areas. At 3 days of age activity of the spinal-cord homogenate is somewhat higher than that in the brain stem, and much higher in the cerebellum, thalamus and cerebral hemisphere. This trend is also obvious at 5 and 10 days of age.

When the tissue was subfractionated, the enzyme activity was present in all fractions and the recoverable activity was present mostly in the microsomal fraction (22–66%) at earlier ages, whereas only 10–20% of the activity was present in the isolated myelin fraction (Tables 2 and 3). However, on a specific-activity basis (μmol/h per mg of protein), myelin fractions were more active than microsomal fractions (Table 4). As maturation of the animals progressed, less of the total hydrolase activity was found in microsomal fractions, but more was associated with myelin. Up to 10 days of age, spinal-cord myelin contained a larger proportion of the hydrolase than in other areas. Adult proportions of the enzyme were present in myelin from cerebellum and thalamic area at 40 and 25 days respectively, whereas in the cerebral hemisphere the enzyme became predominantly myelin-associated much later. In older animals the activity in recovered myelin accounted for 40–60% of the activity of the whole homogenate in all parts of the brain.

The specific activity of 2′:3′-cyclic phosphohydrolase in purified myelin changed with age and varied in the different regions of the central nervous system (Table 4). The activity in spinal-cord and brain-stem myelin was maximal at 15 days of age,

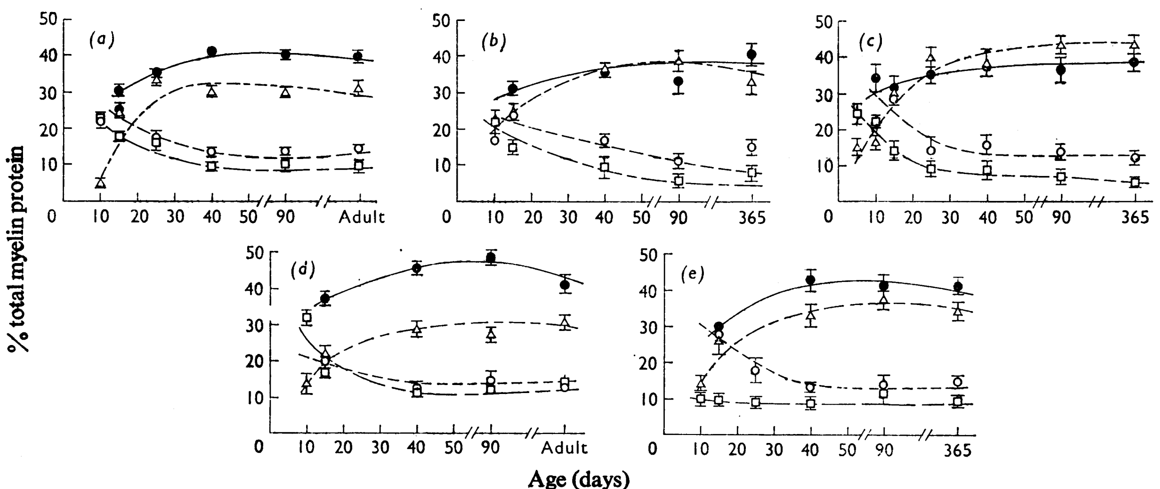

Fig. 2. *Protein composition of myelin from different areas of the central nervous system from developing rats*
Percentage of total dye bound is estimated by triangulation of peaks from densitometric scans of gels separated as in Plate 3. Although basic protein was found to bind 60% more dye than did proteolipid protein, no correction for specific dye binding was applied in our measurement. Bars represent S.D. of four experiments; (*a*) thalamus; (*b*) cerebellum; (*c*) spinal cord; (*d*) cerebral hemisphere and (*e*) brain stem myelin proteins. □, WP; ●, PLP; ○, BL; △, BS. Abbreviations are as in Plate 3.

Table 2. *Distribution and recovery of 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity in subcellular fractions isolated from different regions of developing rat central nervous system*

The enzyme activity is expressed as μmol of product/h per g wet wt. of tissue and recovery as a percentage of the total homogenate activity. Abbreviations: WH, whole homogenate; CR MIT, mitochondria+nuclei; MY, myelin; MIC, microsomal fraction; Supt., supernatant.

		Cerebral hemisphere		Thalamus		Cerebellum		Brain stem		Spinal cord	
Age (days)	Fraction	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)
5	WH	599	—	504	—	625	—	1557	—	2684	—
	CR MIT	102	17.0	128	25.4	144	23.0	269	17.0	493	18.4
	MIC	148	25.0	178	35.0	219	35.0	418	36.4	1267	47.0
	MY	42	7.0	35	7.0	51	8.1	193	12.5	493	18.4
	Supt.	10	3.7	14	2.8	10	1,6	23	1.4	120	4.5
	Recovery	—	52.7	—	70.2	—	67.7	—	67.3	—	88.3
15	WH	6400	—	7920	—	9550	—	17600	—	20750	—
	CR MIT	1008	16.9	990	12.5	1302	13.9	2528	14.5	2973	14.2
	MIC	1513	23.8	1364	17.0	1326	14.3	1845	10.4	3630	16.5
	MY	1012	15.9	1704	22.0	1437	15.0	4218	24.0	6905	31.6
	Supt.	721	11.2	902	11.4	956	10.0	1996	11.3	2083	9.6
	Recovery	—	67.8	—	62.9	—	53.2	—	60.2	—	71.9
40	WH	19800	—	29590	—	24200	—	31900	—	34000	—
	CR MIT	1744	8.8	4435	15.0	3283	13.6	3928	12.3	3849	11.3
	MIC	2749	13.9	4326	14.6	3388	14.0	5690	17.8	7625	22.4
	MY	4262	21.6	15125	51.5	10046	43.5	18070	57.0	18450	54.2
	Supt.	1254	6.4	913	3.4	883	3.6	1032	3.2	2865	8.4
	Recovery	—	50.7	—	84.5	—	74.7	—	90.3	—	96.3

Table 3. *Distribution of activity of 2′:3′-cyclic nucleotide 3′-phosphohydrolase in whole tissue, myelin and microsomal fraction of developing rat central nervous system*
The enzyme activity is expressed as μmol of product/h per g wet wt. of tissue, ± S.D. where applicable, and as a percentage of the total whole homogenate activity. Numbers in parentheses indicate numbers of separate experiments. Abbreviations: WH, whole homogenate; MY, myelin; MIC, microsomal fraction.

		Cerebral hemisphere		Thalamus		Cerebellum		Brain stem		Spinal cord	
Age (days)	Fraction	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)	Activity (μmol/h per g)	Recovery (%)
3 (2)	WH	249; 297	—	334; 337	—	329; 380	—	834; 767	—	1350; 1425	—
	MY	15; 16	5	26; 37	11	21; 35	8	84; 115	12	213; 256	17
	MIC	90	30	122; 184	46	255	60	287; 246	31	520; 715	45
5 (3)	WH	484±58	—	680±112	—	1078±62	—	1360±145	—	2830±214	—
	MY	82±11	17	117±34	17	116±39	12	157±21	11	456±80	16
	MIC	93; 121	22	278; 339	49	340; 267	28	733; 517	44	1678	59
10 (3)	WH	1207±116	—	1338±57	—	2900±280	—	5214±540	—	10700±1540	—
	MY	71±4	6	110±30	9	268±48	9	950±109	18	4340±580	40
	MIC	319: 293	25	846	60	1330; 1100	42	1195; 1028	22	2423; 2331	22
15 (2)	WH	9200; 8910	—	9750; 8250	—	8825; 9900	—	15950; 16500	—	18008; 20680	—
	MY	1100; 900	11	1931; 1457	19	1570; 2062	19	7000; 7862	45	7965; 8132	42
	MIC	1503	17	1364	15	1961	21	3475	21	6726	32
25 (2)	WH	21000; 19000	—	32000; 31900	—	26400; 24200	—	30140; 25000	—	46200; 40700	—
	MY	4827; 3643	21	13200; 12700	41	5183; 4827	20	20000; 14000	62	16450; 15400	38
	MIC	—		—		—		—		—	
40 (2)	WH	17650; 16500	—	30800; 25300	—	17600; 17600	—	27500; 33000	—	36900; 29700	—
	MY	5866; 5574	34	10031; 9035	34	10780; 7180	51	13290; 14666	49	18505; 15400	50
	MIC	—		—		—		—		—	
90 (2)	WH	30000; 22000	—	34150; 29590	—	28600; 30800	—	29150; 33300	—	37250; 31900	—
	MY	11846; 11507	45	−14938	49	16218; 18897	59	17810; 16666	55	23985; 15005	62
	MIC	—		—		—		—		—	
365 (4)	WH	26000±2830	—	28000±1200	—	21300±2116	—	31760±1353	—	31500±2300	—
	MY	11000±1000	42	13800±420	49	10000±1300	48	17510±846	55	20500±1030	65
	MIC	3000; 2420	10	4000; 3700	14	3260; 2000	12	6500; 4500	17	6500; 6230	20

Table 4. *2′:3′-Cyclic nucleotide 3′-phosphohydrolase activity in myelin and microsomal fractions isolated from different regions of developing rat brain*

Results are expressed as μmol of product formed/h per mg of protein, ±S.D. where applicable. Numbers in parentheses indicate numbers of separate experiments.

Age (days)	Cerebral hemisphere		Thalamus		Cerebellum		Brain stem		Spinal cord	
	Myelin	Microsomal fraction	Myelin	Microsomal fraction	Myelin	Microsomal fraction	Myelin	Microsomal fraction	Myelin	Microsomal fraction
3 (2)	30; 26	12	52; 40	14; 21	36; 22	28	94; 115	29; 32	325; 375	55; 66
5 (4)	57±7	17±3	400±62	21±4	154±23	32±4	380±69	50±8	989±130	147±23
10 (3)	250±71	49; 46	450±50	119	1015±108	246; 208	1105±112	195; 178	1307±72	410; 394
15 (3)	1350±133	214	1450±160	268	1605±180	197	1520±90	324	1420±165	835
25 (2)	1430; 1200	—	1520; 1100	—	1512; 1480	—	1230; 1145	—	1180; 1315	—
40 (3)	1680±153	377	1250±130	407	1325±175	460	1282±98	572	1170±110	895
90 (2)	1730; 1410	—	1190; 950	—	1747; 1583	—	1070; 860	—	1090; 990	—
365 (4)	1350±120	340; 280	990±85	360; 430	1203±107	450; 440	836±143	1000; 670	920±130	650; 760

then gradually decreased to a value of about one-half its maximum activity. On the other hand, that in myelin from the cerebral hemisphere and cerebellum reached a peak at about 40 and 15 days respectively and remained high up to 90 days, after which time a slight decrease was observed at 365 days. In myelin from thalamus the activity reached its peak at about 25 days and then gradually decreased.

Discussion

The morphology of the material collecting at the interface between 0.32M- and 0.88M-sucrose, a property characteristic of adult myelin, indicates that in 5-day-old rats recognizable lamellar structures can be seen only in spinal cord and brain stem, but not in other areas. Other amorphous membranous structures seen in the electron micrographs in these fractions and in microsomal fractions may be a primitive precursor of myelin (Plates 1*a*–1*c*). The presence of low amounts of acetylcholinesterase and ATPase (on a percentage-distribution basis) in our myelin preparations from rats of different ages suggests that there is little contamination by microsomal particles (Table 1). The ATPase activity present in our myelin preparation is comparable with that of Norton & Poduslo (1973), who obtained values of 17 and 27 μmol of P_i released/h per g wet wt. of brain in the 6-week-old rat. When the ATPase and acetylcholinesterase activities were calculated on the basis of μmol/h per mg of protein, somewhat higher specific activities were obtained in myelin from earlier stages. This suggests that either myelin is contaminated by microsomal particles, or that this enzyme may be present in glial plasma membranes, as shown by Poduslo (1975) and by Agrawal *et al.* (1974) in the myelin-like fraction. However, electron-microscopic observations of our myelin fraction and the presence of small amount of phosphohydrolase activity in myelin from early stages of development do not suggest that it is grossly contaminated by microsomal particles since this fraction contains most of the recoverable enzyme activity.

Myelin deposition has been found to proceed at an exponential rate in rat brain for at least 1 year (Norton & Poduslo, 1973) and the rate of accumulation of myelin varies in the different regions of the central nervous system (Fig. 1). Thus the maximum rate of myelin accumulation in spinal cord and brain stem as measured by total myelin protein/g wet wt. of tissue was between 10 and 25 days after birth, whereas the rate of deposition did not become maximal until after 15 days in other areas. The rate of deposition of myelin in the cerebral cortex is very slow as compared with other areas.

The myelin fraction isolated from early stages of developing central nervous system contained a com-

paratively large amount of Wolfgram protein (Wolfgram, 1966), particularly in myelin from cerebral hemisphere and thalamic area, regions in which myelin appears latest. Similar observations have been made on myelin isolated from developing brains of different species (Eng *et al.*, 1968; Einstein *et al.*, 1970; Greenfield *et al.*, 1971; Morell *et al.*, 1972; Savolainen *et al.*, 1972; Adams & Osborn, 1973; Zgorzalewicz *et al.*, 1974; Banik *et al.*, 1974; Fishman *et al.*, 1975; Sabri *et al.*, 1975). The appearance of myelin in spinal cord from 2-day-old rats has been demonstrated previously (Waksman, 1959; Kornguth *et al.*, 1966), and the presence of a myelin-specific protein shown in this study in 3-day-old rat spinal cord suggests that this myelin shows some characteristics of mature myelin. With increasing age and central nervous system maturation there was a significant increase in all myelin proteins in all regions, but the relative proportions changed, with a decrease in high-molecular-weight (Wolfgram) protein, except in brain stem, and an increase in the smaller basic protein with a reciprocal decrease in the larger basic protein. These reciprocal relationships may indicate that the smaller basic protein may have been derived from the larger by removal of 40 amino acid residues (Martenson *et al.*, 1972). The changes in proteolipid protein are less striking, with an increase observed in all areas of the brain during the first 4 weeks, then no change thereafter. In the spinal cord the small basic-protein peak as measured by Coomassie Blue staining surpasses that of the proteolipid peak at about 25 days and remains high thereafter. This is in keeping with the observations of others, that the proportion of proteolipid protein is less in spinal-cord myelin than in myelin from other areas of the central nervous system (Morell *et al.*, 1973; Lees & Paxman, 1974; Zgorzalewicz *et al.*, 1974; Smith & Sedgewick, 1975).

The data on the accumulation of 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity presented in the present study and in other previous studies shows a close association between the appearance of this enzyme and myelination (Kurihara & Tsukada, 1968; Banik & Davison, 1969; Olafson *et al.*, 1969; Banik *et al.*, 1974; Sabri *et al.*, 1974; Fishman *et al.*, 1975). Although it is widely believed that myelination in rat brain starts at about 10 days after birth (Uzman & Rumley, 1958), 10–20% of the cyclic phosphohydrolase activity of the whole tissue of 3–5-day-old rats is localized in a subcellular fraction with a density characteristic of myelin even before the typical compacted structures could be seen by electron microscopy.

According to the enzyme criterion, the peak period of myelination in the spinal cord and brain stem is reached before that of cerebral hemisphere, where it appears latest. The later appearance of myelin, as judged by myelin marker enzymes in the cerebral hemispheres, agrees with histochemical and morphological observation (Yakovlev & Lecours, 1967).

The distribution of the cyclic phosphohydrolase activity in subcellular fractions isolated from older rats indicated that it was localized mainly in the myelin, with the remainder in other organelles, which also has been shown by other workers (Kurihara & Tsukada, 1968; Olafson *et al.*, 1969; Banik & Davison, 1969). Although there is variation in recovery (50–96% of the activity; Tables 2 and 3), the unrecovered enzyme may have been lost during the isolation and purification procedure for myelin, a possibility that is especially likely at early stages. However, the subcellular distribution of the phosphohydrolase activity is quite different in young animals from that in adults (Tables 2 and 3). At early stages of development the microsomal fraction, but not myelin, contains much of the recoverable enzyme activity in all areas. A similar observation was made by Sabri *et al.* (1975), who isolated myelin from whole brain as described by Norton & Poduslo (1973). When results are calculated on a specific-activity basis (μmol/h per mg of protein), myelin fractions from earlier ages are more active than microsomal fractions (Table 4). As the animal matures, more of the enzyme is recovered in association with myelin and less with the microsomal fraction. This 'crossover' point is at about 10 days in the spinal cord, 15 days in fractions from brain stem, cerebellum and thalamic area, and later in the cerebral-hemisphere fraction. Thus it is likely that membranes formed in young animals are less well differentiated, and the myelin precursor membranes may possess densities not typical of adult myelin, which may associate with microsomal or other fractions. It is equally likely that at early stages the phosphohydrolase activity is associated with microsomal fraction for a time before it is transferred to myelin through a transitional form of membrane.

The presence of higher phosphohydrolase activity and basic protein in the microsomal fraction obtained in early stages of development suggests that this fraction contains myelin precursor membrane fragments (Plates 2*a*–2*c*) derived from oligodendroglial plasma membrane in the sequence of myelin synthesis from myelin-like membranes to compact myelin. A similar membrane fraction with higher phosphohydrolase activity also has been isolated and a precursor–product relationship between this fraction and myelin has been shown (Banik & Davison, 1969; Agrawal *et al.*, 1970; Greenfield *et al.*, 1971; Agrawal *et al.*, 1974; Sabri *et al.*, 1975; Waehneldt, 1975).

As the membranes are compacted there is a dramatic increase in phosphohydrolase activity, the characteristic proteins appear and the lipid composition typical of mature myelin gradually evolves. These changes correlated well with morphological studies, where an increase in number of lamellae and com-

paction of the myelin sheath during maturation has been demonstrated (Caley & Maxwell, 1968; Banik *et al*., 1974).

The studies reported here have shown that the appearance of morphologically mature myelin in structures where myelination starts earliest may be correlated with a more rapid deposition of myelin protein, earlier appearance of basic protein and 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity, which more rapidly becomes associated with myelin. Those areas in which myelin appears relatively late show delayed appearance of these biochemical parameters. These studies also suggest that the precursor membrane fragments are present in microsomal fraction at an early stage in the process of myelination before the formation of compact myelin.

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