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Myelination in the Developing Human Brain: Biochemical Correlates*

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To delineate the biochemical sequences of myelination in the human brain, we analyzed the protein and lipid composition of white matter in 18 baseline cases ranging in age from midgestation through infancy, the critical period in human myelination when the most rapid changes occur. Three adult cases were used as indices of maturity, and 4 cases with major disorders of CNS myelination (maple syrup urine disease, severe periventricular leukomalacia, idiopathic central hypomyelination, and metachromatic leukodystrophy) were analyzed. Brain samples were obtained ≤24 hours after death. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high performance thin-layer chromatography were used to separate and identify proteins and polar and neutral lipids in an average of 10 sites/brain; computer-based densitometry was used to quantify polar lipids. Biochemical sequences, as manifested by the appearance of the myelin-associated lipids and myelin-specific proteins, closely followed previously described anatomic sequences both temporally and by region, and were identical in all sites sampled: sphingomyelin was followed simultaneously by cerebrosides, MBP, PLP, and nonhydroxy-sulfatide, followed by hydroxy-sulfatide. The onset and tempo of the expression of individual constituents, however, were quite variable among sites, suggesting a wide differential in vulnerable periods to insult in biochemically-specific pathways in early life. Cholesterol ester was transiently elevated during late gestation and early infancy, prior to and around the time of the appearance of cerebrosides, sulfatides, PLP, and MBP. Distinctive lipid and protein abnormalities were detected in idiopathic central hypomyelination and metachromatic leukodystrophy. This study underscores the feasibility of the combined biochemical approaches in pediatric brains and provides guidelines for the assessment of disorders of myelination in early human life.

KEY WORDS: Cholesterol ester; maple syrup urine disease; metachromatic leukodystrophy; myelin basic protein; periventricular leukomalacia; sphingomyelin; sulfatides.

INTRODUCTION

Myelination in the human central nervous system (CNS) is a complex but orderly process which occurs in predictable topographical and chronological sequences that have been carefully defined by anatomic methods (1-9). While human CNS myelination begins as early as 12-14 weeks gestation in the spinal cord (10,11) and continues into the third decade in intracortical fibers of the cerebral cortex (4), the most rapid and dramatic changes occur between midgestation and the end of the second postnatal year (1,2,9), with myelination accounting in large part for the over tripling in brain weight during this period. The process of myelin formation occurs in two, partially overlapping stages in which oligodendrocyte (OL) proliferation and differentiation is followed by myelin synthesis. Major lipid components of the myelin sheath include the galactolipids (cerebrosides and sulfatides) and sphingomyelin (SM) (12). The

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major CNS myelin-specific proteins are proteolipid protein (PLP) and myelin basic protein (MBP), both necessary for membrane compaction (12,13).

By contrast with the anatomic sequences of myelination, our understanding of the biochemical sequences is limited in the human brain, and is based upon analysis in a relatively few cases covering a wide age range and in a few white matter sites (10,11,14-28). Moreover, most previous studies analyzed either lipids or proteins, thereby precluding an integrated picture of how these different but interdependent constituents of the myelin sheath change relative to one another during myelination. In the following report, we present a study of the biochemical correlates of myelination in the developing human brain which builds upon previous studies of the anatomic sequences (1,2), and which focuses upon the simultaneous changes in the major myelin-associated lipids and proteins during the critical period from midgestation through early infancy. Through the analysis of the major lipids and proteins in the same white matter sites in the same brains, this study begins to address the following questions: Are the biochemical constituents of the myelin sheath assembled in the same order, irrespective of the site? Do the myelin-associated lipids and proteins appear simultaneously with one another, or do they follow upon one another in predictable sequences? Are any lipids or proteins expressed only transiently during myelin formation? Is the biochemical composition of fully-formed myelin uniform across all brain regions? Our data can also be viewed as the foundation upon which to build a more complete understanding of the biochemical sequences of human myelination as additional, precious pediatric material is accrued over time.

EXPERIMENTAL PROCEDURE

CNS White Matter Sites Analyzed. The biochemical study reported below builds upon studies of the anatomic sequences of CNS myelination previously performed at Children's Hospital, Boston (1,2). In these anatomic studies, the degree of myelination (0-4) was graded in 62 standardized sites, and was based upon the visual intensity of histological (Luxol-fast-blue) staining compared to an internal standard of "mature" myelin (degree 3) in the inferior cerebellar peduncle. The anatomic studies established eight patterns of CNS myelination in early human life, as defined by the time of the onset of myelination (before or after birth) and the median age at which "mature" myelin (degree 3) was obtained (2), and they helped to classify single and composite fiber pathways as "early", "intermediate", "late", and "very late" (beyond infancy) myelinators.

In the following biochemical study, white matter sites/brain were dissected in a standardized fashion at the same levels in which the anatomic sequences were defined (1,2). These sites included: optic nerve; optic tract; optic chiasm; pyramid (corticospinal tract at level of medulla); middle cerebellar peduncle; frontopontine and temporopontine fibers (level of crus peduncle of midbrain); lateral cerebellar hemisphere; anterior and posterior limbs of the internal capsule; corona radiata; rostrum, body, and splenium of corpus callosum; external and extreme capsules; anterior commissure; cingulum; stria medullaris; Heschl's gyrus; and the central white matter of the frontal pole, posterior frontal lobe (level of body of corpus callosum); posterior parietal lobe (level of atrium); temporal lobe (level of lateral geniculate body), temporal pole, and occipital pole. An average of 10 sites/brain were analyzed; samples were not available for every site in every case.

In this report, data from 3 sites are emphasized to illustrate the biochemical database. These 3 sites begin to myelinate at different times and myelinate with different tempos: 1) posterior limb of the internal capsule, an "early" myelinator in which myelination begins *before* birth and becomes histologically mature (degree 3) by 6 postnatal months; 2) corpus callosum (body), an "intermediate" myelinator in which myelination begins *after* birth and becomes histologically mature (degree 3) by 6 postnatal months; and 3) frontal pole, "late" myelinator in which myelination begins *after* birth and becomes histologically mature (degree 3) by 24 postnatal months (1,2).

Case Selection. For the baseline developmental studies, white matter lipids and proteins were analyzed in the brains of fetuses, premature infants, newborns, and infants without clinical neurological disease. Adult cases were analyzed as indices of maturity for comparison. Four cases with major disorders of CNS myelin (maple syrup urine disease [MSUD], severe perinatal damage of the cerebral white matter with periventricular leukomalacia [PVL], idiopathic central hypomyelination [ICH], and metachromatic leukodystrophy [MLD][29-42]) were analyzed to test the capability of our approach to detect biochemical abnormalities. The fetal brains at midgestation were obtained from elective abortuses from the Department of Pathology, Brigham and Women's Hospital, Boston, MA, with permission of the Human Protection Committee. Brains from cases ranging in age from late gestation through adulthood were obtained primarily from the autopsy services of the Department of Pathology, Children's Hospital and Brigham and Women's Hospital, Boston.

Tissue Preparation. Brains were received within 24 hours of death and stored at −70°C in airtight containers until dissected. Samples from the dissected white matter sites from each brain were homogenized in distilled water to a final concentration of 10% (w/v), and divided into 150-200 μl aliquots for storage at −70°C. The total protein content of each homogenate was determined by Lowry assay with bovine serum albumin as the standard (43).

Determination of White Matter Proteins and Lipids. The white matter samples were dissolved in 5% sodium dodecyl sulfate (SDS) prior to analyses by SDS-polyacrylamide gel electrophoresis (PAGE). Protein aliquots of 40 μg were separated on 8-18% linear gradient

Abbreviations: Polar and neutral lipids: CE, Cholesterol ester; CH, Hydroxy-cerebrosie; CO, Cholesterol; CN, Nonhydroxy-cerebroside; DG, Diglycerides; FA, Fatty acids; MG, Monoglycerides; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; PC, Phosphatidylcholine; PI, Phosphatidylinositol; SH, Hydroxy-sulfatide; SN, Nonhydroxy-sulfatide; SM, Sphingomyelin; ST, Human adult white matter standard (see text); TG, Triglycerides. **Proteins**: LMW.ST, Low molecular weight standard; MBP, Myelin basic protein; PLP, Proteolipid protein; ST, Human adult white matter standard (see text). **Anatomic sites:** AL, Anterior limb of the internal capsule; CCB, Corpus callosum, body; CCS, Corpus callosum, splenium; CG, Cingulum; CR, Corona radiata; DOR, Distal optic radiation; OF, Orbitofrontal central white matter (frontal pole); OP, Occipital pole; P, Posterior parietal central white matter; PL, Posterior limb of internal capsule; PF, Posterior frontal central white matter; ROS, Corpus calosum, rostrum; TL, Temporal lobe (level of lateral geniculate body); TP, Temporal pole.

acrylamide gels (44), and the proteins were stained with Coomassie blue, as previously described (45). Polar and neutral lipids were extracted from the tissue and separated using high performance thin-layer chromatography (TLC), as previously detailed (46). A human "adult" standard was run with each TLC and SDS-PAGE experiment and used for comparison. The standard was prepared from white matter pooled from 3 sites (central white matter of the frontal pole, posterior frontal region, and parietal lobe) in 3 adult brains (Table I: Cases W, X, and Y). For the protein analysis, rabbit CNS myelin was run to indicate the positions of PLP and MBP (data not shown). To confirm the identification of the myelin basic proteins, the separated polypeptides were electrophoretically transferred to an Immobilon-P membrane (Millipore Corp., Medford, MA) using glycine buffer, pH 8.2 (47), incubated with an appropriately diluted, specific antibody (polyclonal rabbit antisera raised against mouse 14 kDa myelin basic protein [provided by Dr. D. Colman] which recognizes the human MBP isoforms), and then incubated with secondary antibody (goat anti-rabbit IgG) conjugated to horseradish peroxidase, which was visualized after staining with 4-chloro, 1-naphthol.

Quantitation of White Matter Polar Lipids. Computer-based densitometry with a MCID imaging system (Imaging Research Inc., Ontario) was used. For comparative purposes in the analysis of developmental changes from midgestation through infancy, the amount of each polar lipid (i.e., density of the individual band on the negative of the photograph of the TLC plate) at each white matter site/brain was considered as a percent of the total polar lipids for that particular site.

RESULTS

Clinical and Autopsy Information. A total of 25 cases, ranging in age from 20 gestational weeks to 71 years, were analyzed (Table I). The cases were divided into 7 age-groups: 1) midgestation (20-21 postconceptional weeks), n = 4 (Cases A-D); 2) late gestation (24-36 postconceptional weeks), n = 5 (Cases E-I); perinatal (39-40 postconceptional wks), n = 2 (Cases J-K); early infancy (1-3 postnatal months), n = 3 (Cases L-N); mid-infancy (7-9 postnatal months), n = 4 (Cases

Table I. Clinicopathologic Information

Case	PC Age (wks)	PN Age (wks)	GA (wks)	Sex	PMI (hrs)	Diagnosis
A	20	0	20	M	4	Abortus
B	21	0	21	M	5	Abortus
C	21	0	21	M	6	Abortus
D	22	0	22	F	7	Abortus
E	24.5	0.5	24	M	12	Prematurity
F	24	1	23	M	18.5	Prematurity: disseminated candidiasis; hyaline membrane disease; acute, mild PVL; intraventricular hemorrhage
G	29	1	28	M	<24	Acute respiratory failure; telencephalic gliosis
H	35	3	32	M	22	Prematurity; acute pneumonia; mild telencephalic gliosis
I	36	1	35	F	24	Congenital diaphragmatic hernia; telencephalic gliosis; PVL; germinal matrix hemorrhage
J	39	1	38	M	24	Maple syrup urine disease
K	40	2	38	F	8	Congenital heart disease; severe perinatal damage of cerebral white matter with PVL
L	46	8	38	M	8.5	Sudden infant death syndrome
M	48	10	38	F	5	Congenital heart disease
N	54	14	40	M	5	Acute pneumonia; mild telecephalic gliosis
O	66	28	38	M	<24	Medium chain acetyl-CoA deficiency
P	70	32	40	F	19	Drowning
Q	70	32	40	M	21	Acute diarrhea and dehydration
R	64	36	28	M	24	Intrauterine cocaine exposure; chronic lung disease; mechanical ventilation; minimal, healed PVL; moderate telecephalic gliosis
S	77	43	34	F	19	Congenital heart disease
T	91	52	39	M	3	Idiopathic central hypomyelination; deafness; congenital cataracts; cirrhosis; club feet
U	—	64	—	M	3.5	Acute respiratory infection; mild telencephalic gliosis
V	—	23 yrs	—	F	18	Metachromatic leukodystrophy
W	—	39 yrs	—	M	18	Acute myocardial infarction
X	—	66 yrs	—	M	18	Systemic cancer
Y	—	71 yrs	—	M	4	Lung cancer

Legend: PC: postconceptional; PN: postnatal; GA: gestational age; wks: weeks; PMI: postmortem interva; -: not available; F: female; M: male.

O-R); and late infancy (11-15 postnatal months), n = 3 (Cases S-U); and adult (23-71 years), n = 4 (Cases V-Y) (Table I). Seventeen of the fetal and infant cases died of miscellaneous causes without clinical neurological disease. Case R, exposed to maternal cocaine in utero, was born prematurely and developed hyaline membrane disease and pulmonary insufficiency that required intermittent mechanical ventilation throughout his life; upon neuropathologic examination, there was mild-to-moderate gliosis of the telencephalic white matter and a single focus of healed necrosis in the periventricular white matter (periventricular leukomalacia), changes consistent with perinatal telencephalic leukoencephalopathy (37). Five of the fetal and infant cases (Cases H, I, J, R, and U) had mild-to-moderate gliosis in the telencephalic white matter, and three (Cases F, I, and R) had periventricular leukomalacia; anatomic evidence for delayed myelination was not apparent. Four of the cases (Cases J, K, T, and V) had major CNS white matter histopathology (Table I): the clinical and neuropathologic findings in these cases are summarized in the Appendix. The 3 adult cases without CNS pathology (Cases W, X, and Y) were used as indices of maturity for comparison. The postmortem intervals were all ≤24 hours (Table I). There was no obvious effect of postmortem interval upon any biochemical parameter analyzed. In the baseline cases, we found little variability in the biochemical profiles within each age-group, despite various causes of death, agonal conditions, postmortem interval, and neuropathologic findings. Data from the cases with major CNS pathology (Cases J, K, T, and V) and from the case with prolonged illness (Case R) were not used in defining the baseline biochemical profiles of CNS myelination.

White Matter Polar Lipids. The phospholipids were the first polar lipids to appear in all CNS white matter sites sampled, and were present well before the initiation of active myelin synthesis and expression of the myelin-associated galactolipids and SM (Figs. 1,2). During gestation, the phospholipids were present in the following amounts relative to one another, from highest to lowest: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) (Figs. 1,2). While all of the phospholipids increased in total amount in individual sites from midgestation through infancy, they remained in approximately the same relative amounts to one another, a relative proportion also retained in adult white matter (Figs. 1,2). PI was initially low and remained relatively constant over the time-period studied. PE, PC, and PS decreased as a proportion of total polar lipids within an individual site as the galactolipids and SM appeared and rapidly increased in amount concomitant with myelin sheath formation (Figs. 1-3). The percent of PC prior to myelin synthesis appeared relatively uniform between sites, as exemplified in the corpus callosum and frontal pole prior to birth (Figs. 1,3). The timing of the change in the percent of PC of the total polar lipids, however, differed markedly among sites, and reflected the timing of myelination of the individual sites (Fig. 3).

The first appearance of the myelin-associated lipids (cerebrosides, sulfatides, and SM) followed the anatomic sequences both temporally and by region (Figs. 1,2). Although the timing of the first appearance of the galactolipids and SM varied among white matter sites, in every site sampled SM preceded the simultaneous appearance of the hydroxy- and nonhydroxy-cerebrosides and nonhydroxy-sulfatide, followed in turn by hydroxy-sulfatide (Figs. 1,2). Initially SM was detected as a single band in all white matter samples, but an additional band subsequently appeared, with variations in the timing dependent upon the site (Figs. 1,2). In the corpus callosum, for example, one band was first detected in the perinatal period, and two bands, in early infancy; in the frontal pole, one band was definitely distinguished in early infancy, and two bands, in mid-infancy (Fig. 1). The second band of SM appeared simultaneously with hydroxy- and nonhydroxy-cerebrosides and nonhydroxy-sulfatide (Fig. 1,2). With increasing age, the total amounts of the galactolipids and SM increased, with the relative amount of nonhydroxy-sulfatide greater than that of hydroxy-sulfatide at every age. The adult percent of total polar lipids was reached for nonhydroxy-cerebroside and nonhydroxy-sulfatide in the posterior limb, corpus callosum, and frontal pole by late infancy, but not for SM in any of these sites (Fig. 3).

White Matter Neutral Lipids. Cholesterol was the major neutral lipid at all ages and in all sites. Cholesterol, as well as monoglycerides and fatty acids, were present in unmyelinated CNS white matter at midgestation in all sites sampled. The adult levels of free fatty acids and monoglycerides appeared increased over pediatric levels, but these neutral lipids did not appear to correlate with the extent of myelination from midgestation through infancy. Diglycerides were not detected in any site at any age (Fig. 4). Triglycerides were not detected in all sites, nor at all ages: they were present, for example, only during gestation in the frontal pole (Fig. 4), but not in the corpus callosum or posterior limb (data not shown). Cholesterol ester (CE) was transiently elevated during late gestation and early infancy, prior to and around the time of the appearance of cerebrosides, sulfatides, PLP, and MBP; thereafter, it was present only in trace amounts or undetectable. CE was found, for example, in greater than trace amounts in the frontal pole

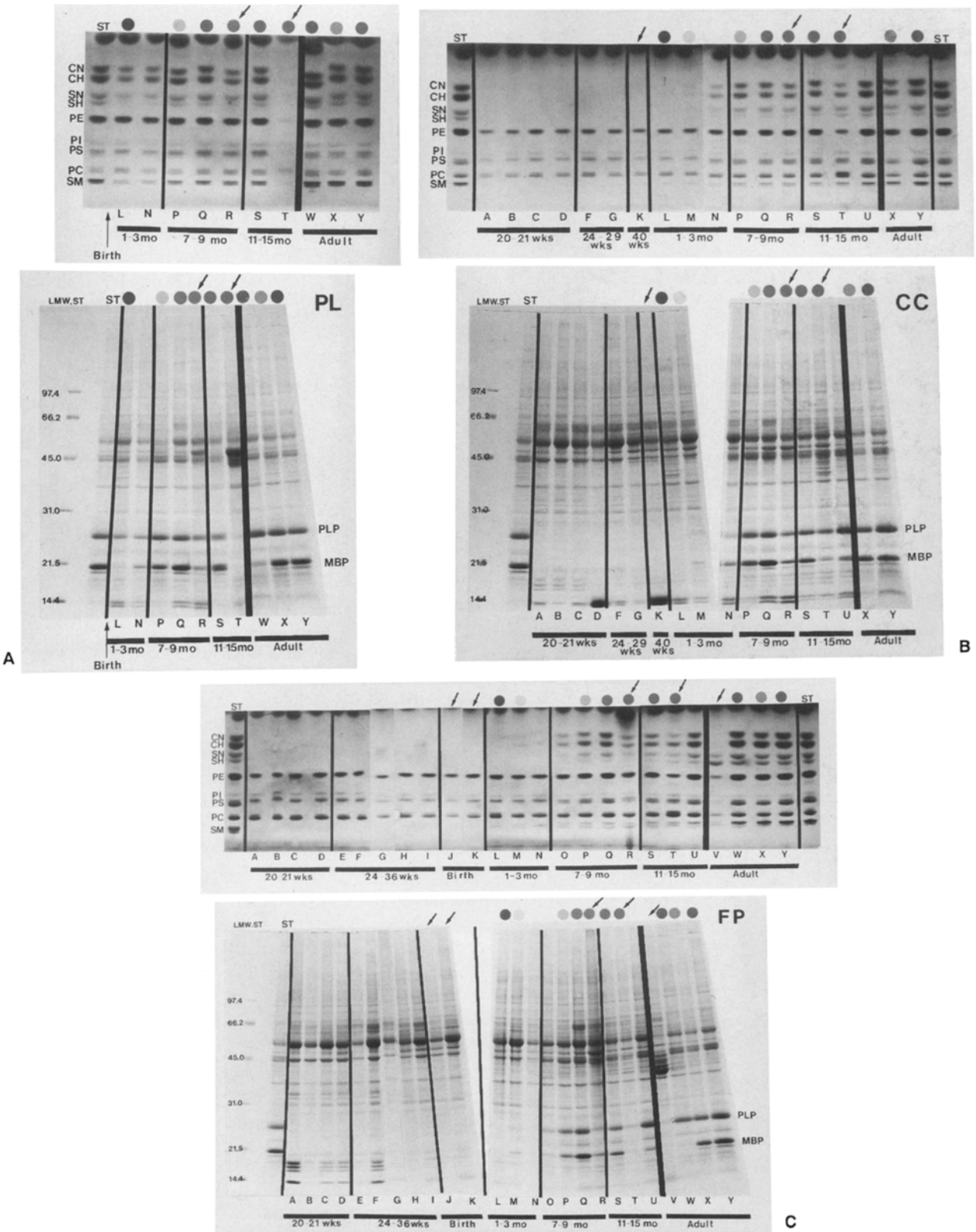

Fig. 1. The developmental sequences of polar lipid and protein expression are illustrated for the posterior limb (PL) (early myelinator) (A); corpus callosum (CC) (intermediate myelinator) (B); and frontal pole (FP) (late myelinator) (C). Each lane (A-Y) represents a single case (Table 1). The cases are arranged from the left in order of increasing postconceptional age. Samples were not available for every site from every case. Color-coded dots mark the cases in which samples were available in the posterior limb, and are provided for each in identifying their positions in the plates for the corpus callosum and frontal pole. Arrows indicate cases with major CNS white matter pathology (Cases J, K, T, V), and prolonged, systemic illness (Case R). Abbreviations are provided in the separate list.

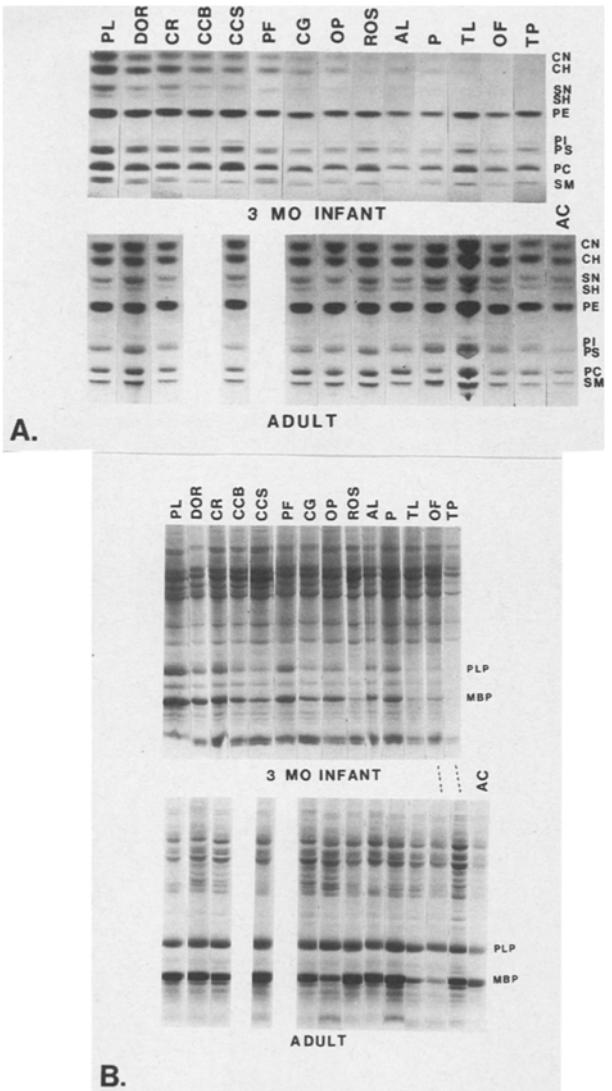

Fig. 2. Polar lipids (A) and proteins (B) are compared between a 3-month-old infant (Case N) and adult (Case W) in selected white matter sites. The sites are arranged from the earliest to latest myelinating site, according to the previously established anatomic sequences (1,2). The appearance of the major myelin-associated lipids (sphingomyelin, cerebrosides, and sulfiatides) and proteins (PLP and MBP) follows relatively closely the anatomic sequences, both temporally and by region. The exceptions in this infant case are the rostrum of the corpus callosum (ROS) which is behind the anatomic sequence, and posterior frontal central white matter (PF), which is ahead. Abbreviations are provided in the separate list.

from late gestation through at least the seventh postnatal month, with only trace amounts in late infancy (Fig. 4). The expression of CE varied considerably among sites in the late fetal and infant brain, but it was never detectable in the brains of midgestational fetuses or adults at any site (Fig. 4). The augmentation of neutral lipids in the chronically ill infant with intermittent mechanical ventilation (Case R) in Fig. 4 is a technical artifact, as it was not present in any of 20 white matter sites, including the frontal pole, which were analyzed from this brain in separate TLC plates.

White Matter Proteins. Like the myelin-associated polar lipids, the major myelin proteins MBP and PLP followed closely the anatomic sequences both temporally and by region (Fig. 1,2). PLP and the 18.5 kDa MBP appeared simultaneously in all sites sampled. In general, the levels of PLP and MBP gradually increased with increasing age. The higher levels of MBP and PLP correlated with lower levels of the high molecular weight proteins (particularly in the 45-66 kDa region). In this initial study, we did not define the developmental appearance of DM-20, nor did we examine the differential expression of the MBP isoforms.

Sequences of the Major Myelin-Associated Lipids and Proteins Combined. The timing of the first (at least trace) appearance of the major white matter lipids and proteins are presented together schematically for the posterior limb of the internal capsule, corpus callosum, and frontal pole for comparison between early, intermediate, and late myelinating sites (Fig. 5). Phospholipids, cholesterol, and monoglycerides were present initially, followed by SM (one band), then cerebrosides, PLP, MBP, nonhydroxy-sulfatide, and SM (two bands) simultaneously, and finally hydroxy-sulfatide. This sequence of the first appearance of the white matter lipids and proteins was essentially the same among all sites sampled, although the timing and rate of expression varied (Fig. 5). Moreover, all the polar and neutral lipids and major myelin-specific proteins that were present in adult white matter were expressed, albeit in variable proportions of adult levels, by the end of infancy (Fig. 1). Comparing previously defined anatomic sequences (1,2) with the biochemical sequences, we found that the major myelin proteins and myelin-associated lipids were detectable before myelin tubules are visible histologically. In the frontal pole, for example, PLP and MBP appeared around 2 postnatal months (Fig. 1), compared to microscopic myelin (degree 1) around 4 postnatal months (1,2). In the posterior limb, which reaches histologically mature myelin in $\leq$6 postnatal months, MBP, PLP, cerebrosides, and sulfatides were already pronounced by the first postnatal month (Fig. 1). On the other hand, many CNS white matter sites were still relatively biochemically "immature" by the end of infancy, although they appeared relatively "mature" with the LFB myelin stain (1,2). When the amount of individual lipid components were

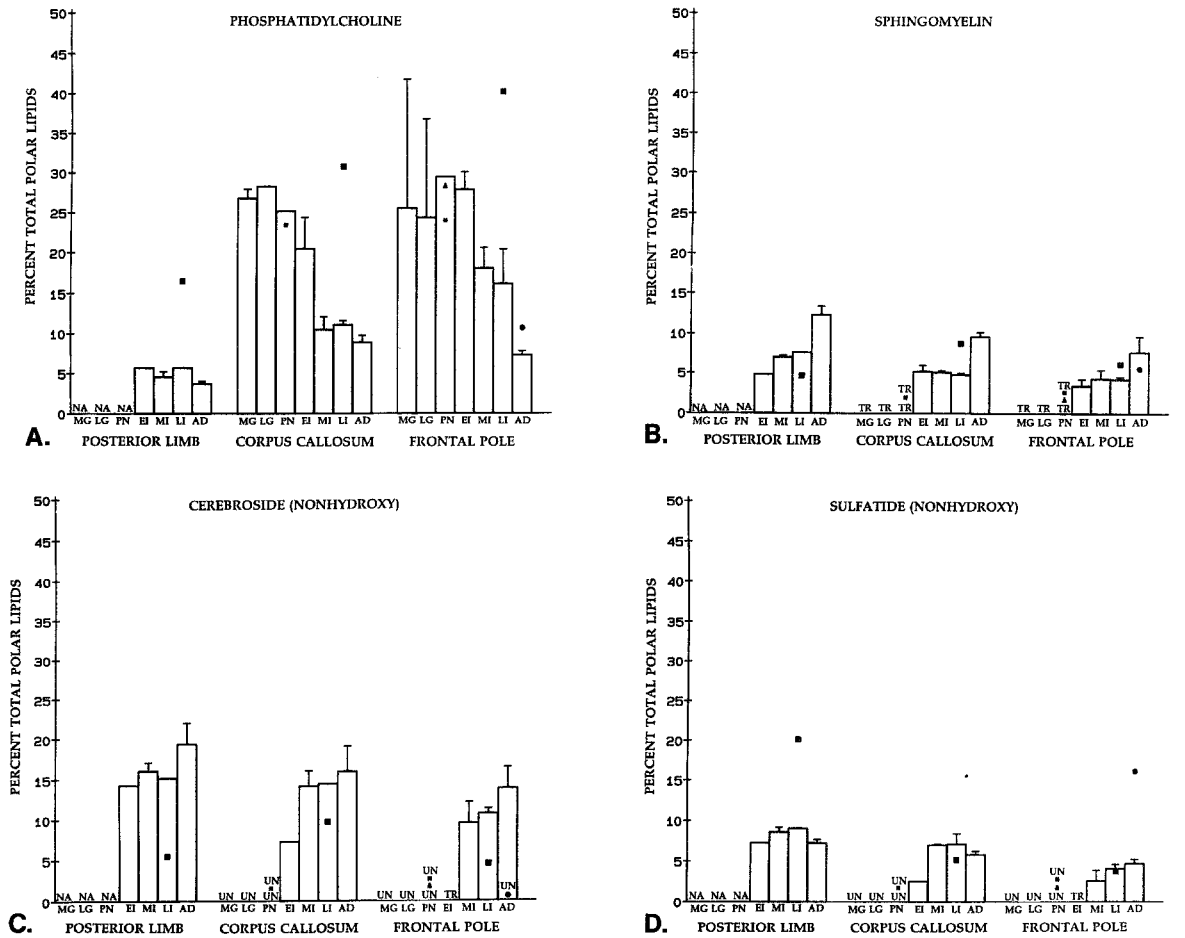

Fig. 3 The developmental changes in the relative amounts of selected polar lipids are compared between the posterior limb of the internal capsule (early myelinator), body of the corpus callosum (intermediate myelinator), and frontal pole (late myelinator). Each polar lipid component is expressed as a percent (±standard deviation) of the total polar lipids for that case at the particular site; the sample size is 1 for a particular age-range in which there is no standard deviation bar. Pre-and perinatal data are not available for the posterior limb. For four of the cases with major CNS white matter pathology, the percent of the selected polar lipids is given in the different sites, with the following symbols representing each case: Case J (MSUD), triangle; Case K (severe perinatal damage of cerebral white matter with PVL), asterisk; Case T (ICH), square; Case V (MCL), circle. A. Phospatidylcholine (PC). B. Sphingomyelin. C. Cerebroside (nonhydroxy). D. Sulfatide (nonhydroxy). Abbreviations: MG, midgestation; LG, late gestation; PN, perinatal, EI, early infancy; MI, mid-infancy, LI, later infancy; AD, adult; NA, not available; UN, undetectable visually in the TLC plate; Tr, trace amount detected visually in the TLC plate.

expressed as the percent of the adult standard, for example, virtually none of the components attained adult levels by the end of infancy, the end-point of this study (data not shown).

In adult white matter, cholesterol, cerebrosides, and PE were the major lipids, followed in decreasing order by sulfatides, sphingomyelin, PC, PS, and PI (Figs. 1,3). PLP and 18.5 kDa MBP were the major myelin-specific proteins. The relative distribution and amounts of lipids and proteins, however, were not always uniform among the "fully mature" white matter sites within individual brains (Fig. 1-3). This point is illustrated by the comparison among multiple sites in one adult brain (Case W), in which, for example, there is a large amount of MBP in the posterior and anterior limbs of the internal capsule and distal optic radiation, but a substantially less amount in the temporal lobe and frontal pole (Fig. 2). Galactolipids were also variable among sites, with relatively high amounts of cerebrosides in the rostrum of the corpus callosum and low amounts in the anterior commissure, and relatively high amounts of nonhydroxy-sulfatide within the distal optic radiation and posterior parietal white matter, compared to low amounts in the temporal pole (Fig. 2). Such variations within different sites within

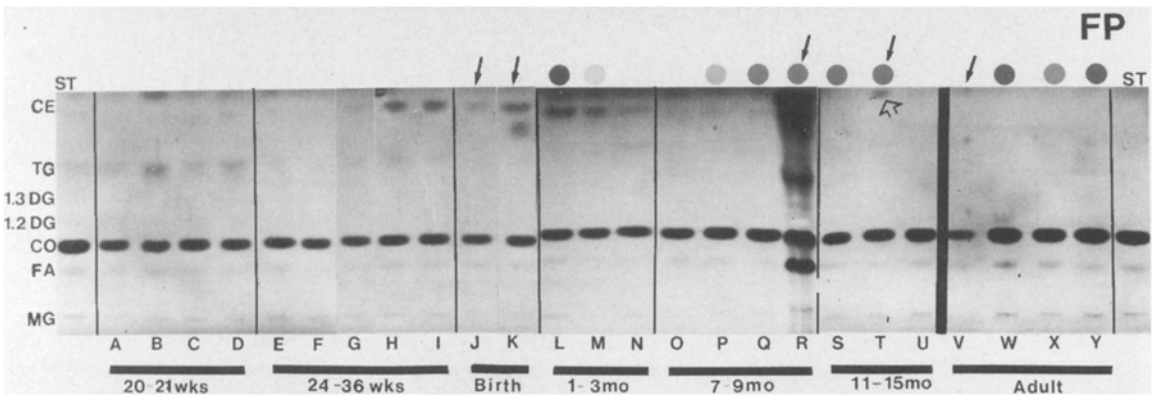

Fig. 4. The developmental sequence of neutral lipids is illustrated in the frontal pole (FP). Each lane (A-Y) represents a single case (Table 1). The cases are arranged from the left in order of increasing postconceptional age. The augmentation of neutral lipids in Case R is a technical artifact (see text). Color-coded dots mark the cases in which samples were available in the posterior limb, and are provided for ease in identifying their positions in the TLC and SDS-PAGE plates for polar lipids and proteins, respectively, in Fig. 1. Solid arrows indicate cases with major CNS white matter pathology (Cases J, K, T, V), and with prolonged, systemic illness (Case R). Open arrow indicates the presence of markedly increased CE for age in this site of the infant with idiopathic congenital hypomyelination. Abbreviations are provided in the separate list.

an individual case, however, were not consistent among cases. In the adult group as a whole, the relative proportions of PC, SM, nonhydroxy-cerebroside, and nonhydroxy-sulfatide were similar between the posterior limb, corpus callosum, and frontal pole (Fig. 3).

Disorders of CNS Myelination

Maple Syrup Urine Disease (Case J): Lipid and protein analysis was performed in the pyramid and frontal pole only. The relative amounts of the phospholipids, including PC (Fig. 3), cholesterol, and monoglycerides appeared comparable to the two other cases in the same age range in these two sites. In the frontal pole, the myelin-associated lipids and proteins were not present, as expected in the perinatal period for this late myelinating site (Figs. 1,3).

Severe Perinatal Damage of the Cerebral White Matter with Periventricular Leukomalacia (Case K): The expression of white matter proteins, polar lipids, and cholesterol was similar to that of other cases in the same age range for available sites (Figs. 1,3). CE, however, was particularly abundant in early, intermediate, and late myelinating sites, but most notably in the cerebral hemisphere, as illustrated in the frontal pole (Fig. 4).

Idiopathic Central Hypomyelination (Case T): There was an overall decrease in the amount of white matter lipids and proteins relative to age, with certain exceptions: PC was markedly increased in virtually all sites sampled, whereas PE appeared decreased (Figs. 1,3). In the frontal pole, for example, PC comprised 40% of the total polar lipids at this site compared to $11 \pm 1\%$ in late-infancy controls (Cases S and U) (Fig. 3), and PE comprised 18% compared to 40% in controls. Two-dimensional electrophoresis demonstrated the presence of plasmalogen (data not shown). CE was abundant in several sites, e.g., frontal pole (Fig. 4), whereas in age-matched controls it was either undetectable or trace. All of the major myelin-specific proteins, including PLP, were present, although at decreased amounts (Fig. 1). The degree of abnormalities did not appear uniform among all of the 15 sites sampled in this case: in the posterior limb, for example, there were virtually no myelin-associated lipids and proteins, whereas they were present, albeit at decreased amounts, in the later myelinating corpus callosum and frontal pole (Figs. 1,3).

Metachromatic Leukodystrophy (Case V): There was a marked decrease in the amounts of all lipids, including cerebrosides, and myelin-specific proteins (Fig. 1,3). The one exception was sulfatides, in which both hydroxy- and nonhydroxy-sulfatides were present and elevated. In the frontal pole, for example, hydroxy-sulfatide comprised 40% of the total polar lipids in this patient, compared to $3 \pm 1\%$ in the 3 adult control cases (Cases W, X, and Y), and nonhydroxy-sulfatide comprised 16% compared to $5 \pm 1\%$ in controls (Fig. 3). Thus, the ratio of nonhydroxy- to hydroxy-sulfatide was reversed in the patient with MLD, with a disproportionately (massive) increase in hydroxy-sulfatide. CE was not detected in any white matter site sampled, despite extensive demyelination and macrophagocytic infiltration. Attempts to isolate myelin from the white matter were unsuccessful due to severe white matter cavitation and demyelination.

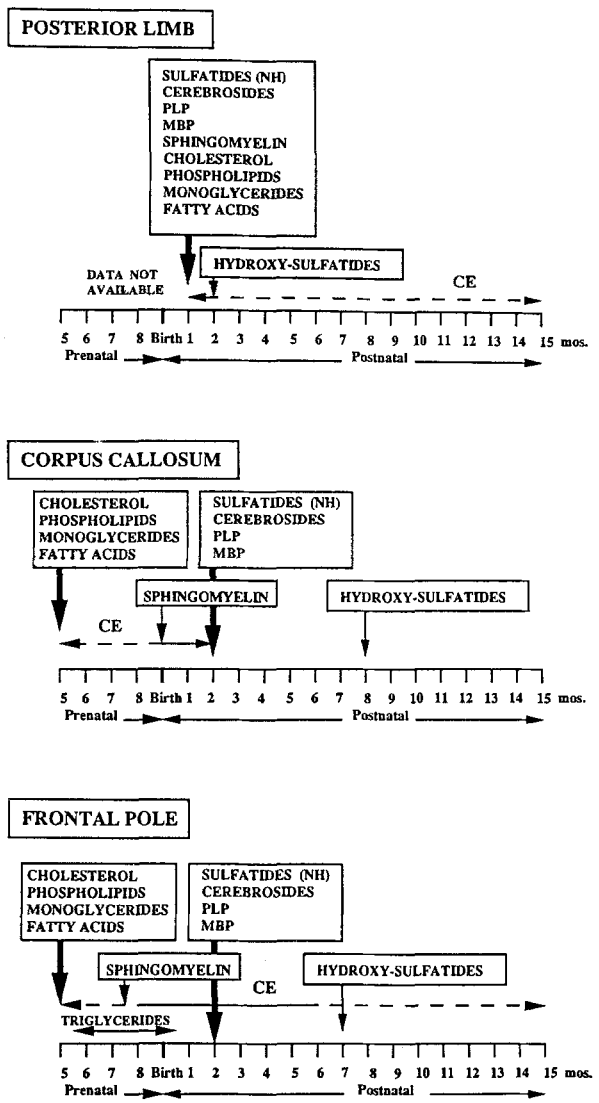

Fig. 5. The sequences of the first appearance of white matter lipids and proteins are presented schematically for the posterior limb, corpus callosum, and frontal pole. In the posterior limb, data were not available from midgestation through the first postnatal month. For cholesterol ester (CE), trace amounts are indicated by the dotted line, and more than trace, by the solid line.

DISCUSSION

This study provides a broad outline of the sequences of some of the major biochemical components of white matter in the developing human brain during the critical period in CNS myelination, and represents a first step towards establishing guidelines for the biochemical assessment of myelin disorders in early human life. Prior to myelin formation, cholesterol, phospholipids, fatty acids, and monoglycerides are abundant in white matter. With the onset of active myelin synthesis, cholesterol and phospholipids increase in amount, and the myelin-associated lipids and myelin-specific proteins appear in a predictable and orderly sequence: SM is followed simultaneously by cerebrosides, MBP, PLP, and nonhydroxy-sulfatide, which are followed in turn by hydroxy-sulfatide. Initially SM appears as a single band, with the subsequent appearance of a second band; the timing of the appearance of the second band is dependent upon the site and its tempo of myelination. These different SM bands reportedly reflect differences in the percentage of very long chain fatty acids, with the single "immature" band containing mainly C_{24}-C_{26} fatty acids, and the "mature" bands, predominately C_{16}-C_{18} fatty acids (27). The biochemical sequence is essentially the same among all sites, and closely follows the previously defined anatomic sequences (1,2), with the biochemical detection of the myelin-associated lipids and proteins preceding that of the histological detection of myelin tubules. Immunocytochemical studies in rodents suggest an even more refined sequence, e.g., sulfatide is expressed first, followed by CNPase, and then, in turn, by galactocerebroside, PLP, and MBP (48-50). Nevertheless, biochemical methods in tissue homogenates provide a readily accessible approach for obtaining basic information about the constituents of myelinating white matter in multiple regions of multiple brains at multiple ages, and for defining specific metabolic defects in pediatric disorders of myelination.

Biochemical Correlates of Human CNS Myelination. Our observations concerning the biochemical composition both of myelinating and adult white matter are similar to those reported by others (12,14,24-28). Previous pediatric studies, however, have focused in general upon the assessment of either lipids or proteins, and in one or two discrete sites or whole brain homogenates (14-28). Thus, the present study expands upon previous information by defining the biochemical sequences of both myelin-associated lipids and proteins in the same brains at different time-points in the critical period of CNS myelination, and in multiple regions in which myelination is known to vary in its time of onset and rate of progression. A potential major discrepancy between this study and previous ones concerns the temporal expression of PLP and MBP. In a study of homogenized spinal cord from human fetuses using SDS-PAGE and immunoblotting, Kronquist et al. found that the appearance of polypeptides for all four isoforms of human MBP preceded the appearance of polypeptides for PLP by 3-4 weeks, an observation dependent upon prolonged exposure of autoradiograms (21). In addition, these investigators found that DM-20 appeared 3 weeks earlier than

PLP (21). In contrast, we found that PLP and the 18.5 kDa MBP appeared simultaneously in all myelinating sites sampled. (The temporal expression of DM-20, as confirmed by immunoblotting, was not addressed in our study.) The temporospatial profiles of the human MBP isoforms and their mRNAs are of major interest in human brain development (21,22) and warrant future analysis in our dataset. An additional finding of interest in this study is the biochemical heterogeneity among different white matter sites within an individual adult brain. This heterogeneity was unexpected as the human myelin sheath is generally considered uniform throughout the brain, both at the biochemical and structural level, once it is completely formed (8). The cellular and molecular basis of this heterogeneity and its functional significance are unknown.

Developmental Expression of Cholesterol Ester. The restricted period of increased CE expression in pediatric brains observed by us and others (24) suggests that metabolic pathways involving CE may be related to myelin synthesis. The synthesis and deposition of cholesterol are particularly active in the brain immediately preceding and during the period of glial proliferation and myelination (56). In rats, a transient increase in CE corresponds exactly to the period of the most active myelination (postnatal days 7-13) (57,58). Although its precise role in myelination is uncertain, CE has been postulated to serve as carrier of fatty acids that are transported to and from other structural lipids during their biosynthesis and degradation (24).

The issue of the developmental expression of CE in human brains is germaine to the controversy in pediatric neuropathology concerning "diffuse fatty change" (31,59-64). This term refers to the presence in developing white matter of lipid-laden cells containing fat droplets demonstrable with neutral lipid stains. In autopsy studies of unselected newborns, fatty change has been reported in over 80% of cases (59): it occurs in both premature and mature infants, but disappears almost completely by the sixth postnatal month (59,61), an observation consistent with the finding in the present study that CE is either present in only trace amounts or undetectable in white matter beyond mid-infancy in virtually all of the sites sampled. A close correlation, however, between fatty change and myelination has not been found in morphological studies (64). The major theories about fatty change are: 1) it represents a normal feature of myelin formation in which neutral lipids are excessive precursors for myelin lipid components and are subsequently exported by macrophages; and 2) it is a pathologic change due to a response of "myelinating glia" to metabolic stress (hypoxia, acidosis) (31,59-64). In a large anatomic study of fetal and infant brains, Leech and Alvord concluded that sudanophilic lipids accumulate to a small degree as a stage of normal premyelin lipogenesis, but that large amounts are abnormal (62). In pathologic conditions of adult white matter, abundant CE, in marked excess of negligible control values, is associated with demyelination, as macrophages phagocytize degenerating myelin, but cannot completely degrade cholesterol and thus store it in the esterified form. In perinatal damage of telencephalic white matter, it is unlikely that the lipid-laden cell are macrophages which store CE as a by-product of myelin breakdown, since myelin sheaths have not yet been formed. In the neonate with severe perinatal white matter damage and PVL (Case K) in this study, abundant amounts of CE were present in early, intermediate, and late myelinating sites in the telencephalon, and correlated with a diffuse and severe infiltration of lipid-laden cells. The presence of abundant CE in telencephalic sites in severe perinatal white matter damage raises the question of an ischemic injury to OL precursors which results in abnormal cholesterol synthesis and/or turnover, thereby causing an excessive accumulation of CE and the potential for impaired myelination. The quantitative profile of cholesterol and CE in human CNS myelination, as well as the relationship of CE to fatty change and perinatal white matter damage, needs to be pursued.

Major Disorders of CNS White Matter. We found marked differences in the total amounts and relative distributions of myelin-associated lipids and proteins in ICH (Case T) and MLD (Case V). The infant with MSUD (Case J) died within a week of birth, and the white matter lesions reported in the brains of older infants dying of MSUD (hypomyelination, spongiform change, and altered lipid and protein profiles [29,30]) were not found. It is likely that these morphologic lesions develop over time postnatally as the result of prolonged exposure to elevated branched chain amino acids and/or their ketoacids, and/or to repetitive hypoxic-ischemic or hypoglycemic insults secondary to metabolic crises. Our case suggests that, prior to birth, CNS white matter may develop appropriately in the protected intrauterine environment. The neuropathologic and biochemical findings in ICH (Case T) appear to be unique and to characterize a new disorder of CNS myelination. (The clinicopathologic details of this case will be reported separately.) A primary failure in the initiation of myelin sheath formation is suggested by the almost complete lack of CNS myelin anatomically in the presence of abundant OLs, the absence of myelin breakdown fragments, inflammatory cell infiltration, and storage products, and decreased amounts of all myelin-associated lipids and

proteins. MLD results from a deficiency in the activity of cerebroside sulfatase (arylsulfatase) which is responsible for cleaving the sulfate radical from sulfatide (41,42). The biochemical findings in our case of MLD (Case V) are similar to those reported by others (38,40,42), and are consistent with the anatomic pathology, with severe demyelination reflected in the overall decreased levels of myelin-associated lipids and proteins, and with the massive accumulation of granular (metachromatic) bodies reflected in the markedly elevated sulfatides. In addition, we found a proportionately massive increase in hydroxy-sulfatide relative to nonhydroxy-sulfatide; the significance of this finding, which to our knowledge has not been previously reported in MLD, is unknown.

Limitation of the Study. A major limitation is that the study is based upon an autopsy population of fetuses, newborns, and infants dying of diverse causes, and while these cases did not have clinical neurological dysfunction, it is possible that the disease or agonal condition may have adversely affected the composition of the white matter samples. This possibility is of particular concern in the perinatal period in which the overwhelming majority of autopsied cases die in intensive care nurseries due to primary pulmonary or cardiac disorders, and have some degree of telencephalic white matter damage (i.e., gliosis and/or PVL) which is presumably hypoxic-ischemic in origin (31,33,59). (Indeed, the increased expression of CE in this age group needs to be considered in this light [see above]). Whereas severe perinatal white matter damage has been linked to delayed myelination in survivors of intensive care nurseries (34-36), effects of minimal-to-moderate damage upon myelination are unknown. The difficulty in obtaining "normal" brains in perinatal and infant cases has been commented upon by previous investigators (1,2,20,28); moreover, several investigators have attempted to chose "normal" brains for analysis based upon individual biases about normality which may be incorrect (1,2). In the present study, we have made no assumptions about normality, and we caution that the data not be interpreted as "normative standards", but rather, utilized only as guidelines. Given these caveats, we nevertheless found that the intensity and relative distribution of the major white matter lipids and proteins were remarkably constant among cases within defined developmental time-points, irrespective of the cause of death or agonal condition, suggesting a baseline biochemical profile. Moreover, deviations from this baseline profile were obvious in profound disorders of CNS white matter, e.g., ICH and MLD. In order to detect subtle biochemical differences in a particular disorder, a large, age-matched database is needed that accounts for unavoidable autopsy variables (e.g., disease and agonal states, postmortem interval) in the pediatric population, as well as developmental and inherent biologic variability. The primary aims of this study are to provide an outline of the biochemical sequences of CNS myelination in a population of autopsied fetuses and infants, and to underscore the implications of these observations for further analysis in pediatric disorders of myelination.

CONCLUSION

We report several biochemical correlates of myelination in the developing human brain. The marked heterogeneity in lipid and protein composition in different sites within a relatively short time-span (midgestation through infancy) suggests that a prerequisite for biochemical comparisons between pediatric cases with white matter disease and controls is rigorous matching, not only by age, but also by site. The variability in the appearance (before or after birth) and tempo (slow or fast) of expression of the individual lipid and protein components further suggests that there is wide differential in the vulnerable periods to insult in biochemically-specific pathways in myelinating white matter. An inborn or environmental insult to the metabolic pathways of cholesterol or the major phospholipids, for example, is likely to result in global and serious impairments in myelination at any time-point during gestation or infancy, given the ubiquity and abundance of these lipids in developing white matter as early as midgestation onward. In contrast, the consequences of an insult to galactolipid metabolism are likely to be highly dependent upon the timing and site, such that prior to birth, a galactolipid-specific insult would probably have little or no effect in telencephalic sites, whereas after birth, it could be devastating as myelin sheath synthesis begins and rapidly progresses within these regions. The biochemical heterogeneity among myelinating sites, as demonstrated in this study, likely contributes substantially to the regional variability and complexity of the histopathology of many inborn and acquired disorders of CNS white matter in the fetal, perinatal, and infant periods.

APPENDIX

Clinicopathologic Summaries of Cases with Major CNS White Matter Disorders

Maple Syrup Urine Disease (Case J): The patient was a 6-day-old boy who was found unexpectedly dead

in the crib. The pre- and perinatal history was unremarkable. Newborn screening analysis, completed one day after the infant's death demonstrated markedly elevated blood leucine levels, diagnostic of MSUD. Neuropathologic examination revealed mild, nonspecific gliosis in white matter of telencephalon and cerebellum. The degree of myelination appeared appropriate, and definite spongiform lesions were not present in sites which myelinated before birth. There were acute neuronal and glial changes, consistent with agonal hypoxia-ischemia.

Severe Perinatal White Matter Damage with Periventricular Leukomalacia (Case K): The patient was a 12-day-old girl with complex congenital heart defects (hypoplastic left heart syndrome). She was the full-term, 2929 gram product of a primigravida mother. The pregnancy was complicated by maternal hypertension. The delivery was normal. On the second day of life, the infant developed respiratory distress and cyanosis, and the cardiac defects were diagnosed. She underwent palliative cardiac surgery on the fifth day of life: deep hypothermic cardiopulmonary bypass with cardiac and circulatory arrest was performed. The postoperative course was complicated by sepsis, bradycardia, hypotension, and acidosis. She died two days following surgery. Neuropathologic examination was remarkable for acute periventricular leukomalacia (focal coagulative necrosis) in the anterior frontal lobe, extensive gliosis in the cerebral and cerebellar white matter, and mild brainstem gliosis, all lesions consistent with perinatal hypoxia-ischemia. Myelination did not appear delayed by histological (Luxol-fast-blue) staining. Lipid-laden macrophages, as demonstrated with the ORO stain on frozen, unfixed tissue sections, were numerous throughout the telencephalic white matter.

Idiopathic Central Hypomyelination (Case T): The patient was a 13-month-old boy with developmental delay, congenital cataracts, deafness, club feet, cirrhosis, and failure-to-thrive. The family history was negative for neurological or metabolic disease. All tests for known inborn errors of metabolism were negative, including for peroxisomal disorders. Neuropathologic examination revealed severe hypomyelination and gliosis of the cerebrum, cerebellum, brainstem, and spinal cord. There was no obvious deficiency of oligodendrocyte number; myelin breakdown, macrophagocytic infiltration, and/or storage material were not present. Peripheral myelin was morphologically intact.

Metachromatic Leukodystrophy (Case V): The patient was a 23-year-old woman with the juvenile variant of metachromatic leukodystrophy. She presented at 4 years of age with learning difficulties and ataxia, which were followed by rapidly progressive dementia, seizures, and severe motor impairment. She received institutional care from 5 years of age until her death, a total of 18 years. Neuropathologic examination revealed severe demyelination, including with cavitation, in the white matter of the cerebrum, cerebellum, brainstem, and spinal cord, associated with oligodendrocyte loss and gliosis. Granular bodies, consistent with sulfatide accumulation, were present throughout white matter and selected gray matter regions. Neuronal storage was noted in certain populations, and there was severe cerebellar and thalamic atrophy. The peripheral nerves were severely involved.

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