The Lipid Composition of Rat Brain Myelin and Subcellular Fractions during Development

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1. The lipids of whole brain and subcellular fractions of the rat were analysed during development. 2. The deposition of cholesterol occurred in two phases, one related to increasing wet weight of the brain and the second to myelination. Cerebroside accumulation was related only to myelination. 3. The composition of myelin isolated from 12-day-old rat brain was different in some respects from that of the adult. In the former there was an increase of phospholipid in relation to cholesterol and a marked deficiency in cerebroside. 4. It is suggested that early myelin is extruded glial plasma membrane, which only later becomes mature myelin.

During the development of nervous tissue relatively large amounts of lipid are accumulated, particularly in the white matter of the central nervous system (Robins & Lowe, 1961). Though changes in the lipid composition of the growing brain in various species have been studied by many investigators, it has not been easy until recently to relate these analyses to the anatomical events occurring during development. The introduction of reliable methods for the isolation of relatively homogeneous subcellular fractions and myelin from brain (see Whittaker, 1965; Gregson, 1965) has now made it possible to relate changes in chemical composition to each of the fractions isolated by centrifugal techniques. The present paper is concerned with examining such changes in lipid composition of subcellular fractions of developing brain, with particular emphasis on differences in the chemical composition of the maturing myelin sheath.

METHODS

Albino Wistar rats of either sex and of various ages were used throughout. Ether-anaesthetized rats were killed by exsanguination, and the brains quickly removed and weighed immediately in pre-cooled beakers; young rats up to 25 days were rapidly decapitated without prior anaesthesia. The brains were then either immediately homogenized with 20 ml. of chloroform-methanol (2:1, v/v)/g. fresh wt. to extract the total lipids (Folch, Lees & Sloane-Stanley, 1957) or prepared for subcellular fractionation.

Preparation of subcellular fractions. After being weighed, the brains were suspended in 0.32M-sucrose containing EDTA (1mM) by using a Potter-Elvehjem type of homogenizer (Aldridge, Emery & Street, 1960; Webster & Smith, 1964). Subcellular fractions were prepared as described by Cuzner, Davison & Gregson (1965). After the nervous tissue

had been suspended in sucrose solution, the final volume was adjusted to contain approx. 1 g. wet wt. of whole tissue/10 ml. A portion of the suspension was removed for lipid analysis and the remainder fractionated in the MSE Superspeed 25 centrifuge at 2° at approx. 10000g-min. to remove nuclei and cell debris. The nuclei and cell debris were washed once and recentrifuged. The combined supernatant and wash were then spun at 13500g for 15 min. in the angle head (8×50 ml.) to give a crude mitochondrial fraction, containing mitochondria, detached nerve endings and the bulk of the myelin. The supernatant obtained after sedimentation at 13500g was further centrifuged at 30000g for 60 min. The resulting pellet constitutes the microsomal fraction.

Preparation of myelin, nerve endings and mitochondrial fractions. To prepare myelin from the brains of young rats in sufficient quantity for analysis, up to five brains were used for each fractionation. For animals over 40 days of age, one brain was sufficient for the preparation of adequate amounts of myelin. The crude mitochondrial pellet from every fractionation was treated identically. The pellet was always resuspended by homogenization in approx. 5–6 ml. of 0.32M-sucrose. An equal volume of 1.2M-sucrose was added to give an approximate final concentration of 0.8M. This suspension was layered in two 20 ml. polypropylene tubes over 9 ml. of 1.2M-sucrose, and 3 ml. of 0.32M-sucrose was placed on top of the suspension. The tubes were spun in the MSE Superspeed 50 centrifuge, with the 3×20 ml. swing-out head, at 53500g for 60 min. (Whittaker, 1965). The myelin collected at the top interface, the nerve endings collected at the second interface and the mitochondria formed a firm pellet at the bottom of the tube. The supernatant above the mitochondria was removed with the nerve endings. The myelin was removed in a minimal volume of sucrose solution and diluted with 10–15 vol. of distilled water. After storage at 0° in an ice bath for 20 min. the 'water-shocked' myelin fraction was recentrifuged in the angle head of the MSE 25 centrifuge at 30000g for 30 min.

Purification of myelin fraction. The myelin from five

Table 1. *Lipid composition of developing rat brain*

Experimental details are given in the text. The results are expressed as μ moles of lipid/whole brain. Brains from five 1-day-old rats were pooled to obtain a lipid extract. This number was progressively reduced to one or two brains for each lipid extract, as brain weight increased.

Lipid	Age (days) ...	1	2	3	4	7	10	12	14	16	20	30	36	50	70	100	Adult
Cholesterol	Brain wt. (g.)	0.25	0.27	0.38	0.53	1.00	1.13	1.21	1.25	1.31	1.55	1.70	1.72	1.79	1.87	1.989	6
Cerebroside	No. of analyses	2	3	2	2	3	1	2	3	3	1	2	3	3	3	2	6
Total phospholipid		2.42	2.95	3.53	4.20	16.00	26.00	26.40	26.90	31.50	51.00	66.10	87.00	95.00	98.00	96.00 $\pm$ 3.60	22.20
Serine phospholipid		0	0	0	0	0.18	0.21	0.52	1.25	2.62	8.00	12.05	15.60	19.90	20.00	119.00 $\pm$ 14.00	13.80 $\pm$ 2.30
Phosphatidylinositol		7.40	7.00	11.8	16.40	33.60	40.09	50.00	53.00	60.20	65.00	81.60	120.00	120.00	120.00	6.00 $\pm$ 1.00	5.50 $\pm$ 1.10
Cardiolipin		0.69	0.67	1.14	1.33	3.47	—	5.70	—	5.94	—	12.00	—	—	—	7.16 $\pm$ 1.46	42.80 $\pm$ 4.70
Sphingomyelin		0.29	0.28	0.48	0.59	1.29	—	1.73	1.38	2.45	—	4.80	—	—	—	48.80 $\pm$ 1.62	26.50 $\pm$ 3.58
Lecithin		—	0.18	—	—	0.71	—	1.03	1.54	2.85	—	—	—	—	—	—	—
Ethanolamine phospholipid		0.17	0.15	0.19	0.25	0.61	—	1.50	1.47	2.06	—	5.18	6.50	—	—	—	—
Total plasmalogen		2.47	2.92	4.00	6.00	12.90	—	16.45	16.40	16.70	—	28.30	45.20	—	—	—	—
		2.27	2.05	3.25	5.03	11.10	14.51	18.20	18.40	19.70	—	33.00	46.40	—	—	—	—
		0.65	0.55	0.98	1.58	3.93	—	5.70	5.30	7.85	—	16.25	21.70	—	—	—	—

separate adult and two separate 12-day-old rat brain fractionations was further purified (Autilio, Norton & Terry, 1964). After washing and recentrifugation, the myelin pellet was resuspended in approx. 5 ml. of 0.32M-sucrose. A discontinuous gradient, consisting of 3 ml. each of 0.8M-, 0.7M-, 0.6M-, 0.5M- and 0.4M-sucrose, was prepared in a 20 ml. centrifuge tube and the myelin suspension was layered to the top of the gradient. The tube was spun at 53500g in the 3 $\times$ 20 ml. swing-out head of the MSE 50 centrifuge for 60 min. The adult myelin separated into three distinct layers, plus a pellet, whereas the myelin from a young brain formed only one narrow distinct layer and one wide diffuse layer, plus a pellet. The various layers of both young and adult rat brain myelin were examined by electron microscopy (Cuzner *et al.* 1965).

Preparation of lipid extracts. Lipid extracts of whole brain were prepared by homogenizing the tissue with 20 ml. of chloroform-methanol (2:1, v/v)/g. fresh wt., according to the method of Folch *et al.* (1957). Subcellular fractions were suspended in water and each volume was extracted with 20 vol. of chloroform-methanol (2:1, v/v). Myelin fractions were freed of proteolipid protein by the method of Webster & Folch (1961). Lipid analyses were determined by quantitative thin-layer chromatography as described by Davison & Graham-Wolfaard (1964) and Cuzner & Davison (1967).

RESULTS

The changes in lipid composition of the whole rat brain at different ages after birth are shown in Table 1. Gangliosides, sulphatides and polyphosphoinositides were not analysed. The results

Fig. 1. Changes in the rate of cholesterol accumulation and increase in wet weight in the rat brain during development. The results are shown as percentages of the maximum rate of cholesterol deposition (●) and rate of increase of brain wet weight (□) per day.

Table 2. *Lipid analysis of developing rat brain subcellular fractions*

Experimental details are given in the text. The results are expressed as μ moles of lipid/whole brain. The numbers of brains used in the fractionation are indicated in parentheses.

Age (days) ...	5	10	16	21	30*	70	Adult*
Mean brain wt. (g).							
(no. of brains) ...	0.40 (5)	1.0 (4)	1.25 (3)	1.39 (3)	1.55 (2)	1.79 (1)	1.989 (6)
No. of analyses ...	1	2	2	3	2	3	6
Fraction							
			Cholesterol (μ moles/whole brain)				
Whole homogenate	3.53	15.34	29.62	34.00	54.00	95.00	95.0
Nuclei	—	0.44	0.92	1.05	—	4.17	6.5
Myelin	0	1.47	3.20	5.50	15.4	33.80	34.0
Nerve endings	0.22	3.86	6.72	11.20	—	18.30	—
Mitochondria	—	0.50	0.82		—	2.22	1.3
Microsomes	1.92	5.10	9.80	13.20	15.3	10.40	14.6
Supernatant	—	4.65	8.35	7.05	—	7.70	—
			Cerebroside (μ moles/whole brain)				
Whole homogenate	0	0.193	1.320	2.68	7.3	20.30	22.20
Nuclei	—	0	0.021	—	—	0.80	1.40
Myelin	0	0.077	0.321	1.00	4.5	—	11.80
Nerve endings	—	0.020	0.064	—	—	1.14	—
Mitochondria	—	0.006	0.028	—	—	—	0.06
Microsomes	—	0.040	0.253	0.47	1.2	0.71	2.03
Supernatant	—	0.056	0.182	—	—	0.85	—
			Total phospholipid (μ moles/whole brain)				
Whole homogenate	11.80	28.60	46.35	65.00	72.0	125.0	118.00
Nuclei	—	0.86	1.78	2.18	—	8.5	—
Myelin	0	2.07	4.47	8.80	13.9	30.2	32.00
Nerve endings	—	7.45	9.50	10.60	—	29.2	—
Mitochondria	0.42	1.23	2.20	6.40	—	5.9	4.44
Microsomes	2.80	9.55	15.50	18.50	21.9	14.4	17.67
Supernatant	—	7.70	12.90	11.60	—	14.4	—
			Sulphatide (μ moles/whole brain)				
Myelin	—	0.012	0.044	—	—	—	2.3
			Lecithin (μ moles/whole brain)				
Myelin	—	0.86	1.20	3.27	—	10.4	8.4
			Ethanolamine phospholipid (μ moles/whole brain)				
Myelin	—	—	1.180	3.57	—	—	14.4

* Microsome fraction spun at 100000g for 2 hr.

show the striking increase in cholesterol content in the brain during the period 7–10 days *post partum*; a less marked accumulation of phospholipid occurs during the same time-interval. As myelination proceeds there is a steady increase in lipid, until after 50 days no change in phospholipid content was recorded and subsequent increase in cholesterol content was minimal. Data from Table 1 and unpublished observations (A. N. Davison & N. A. Gregson) on changes in total cholesterol content (μ moles) and wet weight (g.) of the developing rat brain were used to construct smooth curves of accretion in relation to increasing age. The rate of increase of cholesterol or wet weight of brain was determined from each graph for each day. The maximum rate of increase per day was taken as 100 and all other increments are expressed as a percentage of this maximum rate (Fig. 1). However, the

appearance of cerebroside in the rat brain is delayed, and it is only at about 20 days *post partum* that the rate of deposition becomes comparable with that of the other lipids.

In the next series of experiments brain homogenates were prepared from rats of different ages and the suspensions were separated by the centrifugal technique described above. In this paper myelin has been strictly defined as material collecting at the interface between 0.32M- and 0.8M-sucrose solution as described in the Methods section. It was not possible to isolate myelin from rats up to 9 days of age, although thereafter myelin was obtained in increasing amount with increasing age. Analyses of the various brain subcellular fractions for cholesterol, total phospholipid and cerebroside, expressed as amounts per whole brain, are shown in Table 2. During the period studied

there is in general an early increase in lipid content of all fractions, but this increase is sustained only in the myelin fraction. However, these changes with age are smaller when expressed per g. wet wt.; for example, there appears to be minimal change in mitochondrial lipid/g. wet wt. of brain during development. This is consistent with the observation of Gregson & Williams (1966) that there is no increase in mitochondrial number/g. wet wt. during development. There is a substantial change in myelin lipid with increasing age; this is shown in Fig. 2, where the relationship between cholesterol content/g. wet wt. of brain and age for microsomal and myelin fractions is recorded. The phospholipid composition of both fractions is also shown in more detail in Table 2. The molar ratio of total phospholipid to cholesterol in myelin decreases with age and the cerebroside content increases markedly. In the microsomal fraction of the brain of the young rat quantities of cerebroside are found comparable with that in myelin, but later in development relatively more cerebroside is found in myelin than in microsomes.

Analysis of myelin subfractions. Since analysis of the whole myelin fraction harvested from young rat brain is deficient in cerebroside, further attempts were made to subfractionate the sample in comparison with that obtained from adult brain. Samples of myelin from 12-day-old and adult brain were layered over a discontinuous sucrose gradient and centrifuged at 53500g for 1 hr. Four subfractions were obtained from adult myelin and three from the 12-day-old rat myelin (Fig. 3). Electron micrographs were prepared for each subfraction.

All fractions from adult rats appeared to contain predominantly myelin. Lipid analysis of the four fractions of adult myelin shows that only fraction 4 does not have the typical composition of a purified mature rat myelin. These are preliminary results, but they illustrate the possibility of the further subfractionation of myelin (see Table 3).

DISCUSSION

The data presented in this paper provide a detailed comparison of the rates of accumulation of different lipids in the whole rat brain and its subcellular fractions during development. Examination of the rate of deposition of cholesterol with age shows that this process apparently occurs in two stages (Fig. 1). The first stage precedes the first formation of myelin rings and the second occurs at the same time as myelination. Thus accumulation of cholesterol in the brain increases dramatically at 7–10 days *post partum* in the rat, and this period coincides with the period of maximum increase in brain wet weight (Fig. 1). Subsequently the rate of

Fig. 2. Changes in cholesterol content of myelin and microsomes prepared from rat brains during development. Results calculated are for microsomal (●) and myelin (○) fractions isolated as described in the Methods section. The ordinate indicates μ moles of cholesterol/g. equivalent of the original wet wt. of brain used for each preparation (e.g. in 10-day-old rats myelin contains 1.47/1 μ moles of cholesterol/g. wet wt. and microsomes contain 5.10/1 μ moles of cholesterol/g. wet wt.; see Table 2).

Fig. 3. Representation of myelin subfractions.

cholesterol deposition decreases up to about 15 days, after which accumulation may be related to increasing myelination. Though there are less marked changes in the rate of accretion of phospholipid, accumulation of cerebroside is closely related to the deposition of myelin. A similar pattern of change has been seen in mouse brain (Folch, 1955; Uzman & Rumley, 1958) and in rabbit brain (Edgar, 1957; Davison & Wajda, 1959). Further analyses of these changes in lipid composition at the

Table 3. Analysis of lipids of myelin subfractions

Preparation	Subfraction	No. of analyses	Cholesterol (μ moles/brain)	Lipid molar ratio		Lipid molar ratio	
				Cholesterol:phospholipid	cerebroside	Total phospholipid:lecithin:ethanolamine phospholipid	
12-day-old rat myelin	1	2	1.430	100	: 84	: 17	100 : 25 : 48
	2	1	0.290	100	: 107	: 11	100 : 38 : 33
	3	1	0.082	100	: 330	trace	100 : 34 : 51
Adult myelin	1	2	7.9	100	: 90	: 30	
	2	2	20.2	100	: 80	: 26	
	3	2	9.6	100	: 86	: 26	
	4	2	3.1	100	: 94	: 13	

The myelin obtained from the crude mitochondrial pellet was relayed in 0.32 M-sucrose over a discontinuous sucrose gradient from 0.8 M down to 0.4 M, and centrifuged at 53 500 g for 1 hr. Five 12-day-old rat brains were pooled for fractionation, but for each adult fractionation only one brain was necessary.

subcellular level show that the rapid initial accumulation of cholesterol and phospholipid in the newborn brain is reflected in the increasing lipid content of the subcellular fractions. After this early phase of growth, changes in the lipid spectrum of the various organelles is slight, particularly if the results are expressed per g. wet wt. (Fig. 1). The compositions of the individual subcellular fractions are similar, a finding in agreement with the views of Abdel-Latif & Abood (1965) on the common lipid ontogenesis of the subcellular fractions of the brain. The slight increase in lipid content of the microsomal fraction during the period 15–20 days *post partum* may be related to the active lipid synthesis occurring within the central nervous system at this time. Uzman & Rumley (1958) suggested that there is an accumulation of lipid in the brain immediately before the onset of myelination, but the present results showed no evidence during this time of excess of lipid other than that associated with brain growth.

Changing composition of myelin from the developing rat brain. Myelination commences in the rat at about 10 days *post partum*, when rings of myelin can be seen by light microscopy and electron microscopy. The process appears histologically to be most active in the 15–20-day-old rat brain. It is not until animals are 10 days old that myelin can be isolated; it then increases with age of the rat up to about 70 days *post partum*. The myelin fraction isolated from rats between 10 and 25 days old appears histologically similar to that of the adult, but the lipid composition is different. There is a relative deficiency of cerebroside and rather more phospholipid than in the adult (Davison, Cuzner, Banik & Oxberry, 1966; Banik & Davison, 1967; Horrocks, Meckler & Collins, 1966). Also, the fatty acids obtained by alkaline hydrolysis of total lipid from ‘early’ myelin are richer in C₁₆ and saturated fatty acids than are the fatty acids obtained from adult myelin (N. L. Banik & A. N. Davison, unpublished work). The low cerebroside content, the ratio 0.62–0.71 mole of cholesterol/mole of phospholipid and the prevalence of shorter-chain fatty acids is typical of cellular membranes other than myelin (O’Brien, 1965; Ashworth & Green, 1966; Benson, 1966), including, possibly, the plasma membrane of the oligodendroglial cell (Davison *et al.* 1966). It has therefore been suggested that the material behaving like myelin isolated from the developing rat brain is composed of mature myelin and also glial cell membrane.

This hypothesis is in conformity with the view that myelin is initially formed by the wrapping of oligodendroglial membrane about the nerve fibre (Bunge, Bunge & Pappas, 1962) and that this phase of myelinogenesis is shortly followed by the accumulation of cerebroside and an increased

cholesterol/phospholipid ratio. This concept harmonizes with the observation of morphological differences seen in the early stages of myelination in tissue culture (Speidel, 1964; Peterson & Murray, 1965), in electron microscopy (De Robertis, Gerschenfeld & Wald, 1958) and in chemical and histochemical changes in the developing optic nerve (N. L. Banik, M. J. Blunt & A. N. Davison, unpublished work).

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