Turnover of Brain Mitochondrial Membrane Lipids

By M. LOUISE CUZNER, A. N. DAVISON AND N. A. GREGSON*

Department of Biochemistry, Charing Cross Hospital Medical School, London, W.C. 2

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1. The turnover of lipids, of myelin and other brain subcellular particles has been studied in double-labelling experiments on intact rats. 2. Overall metabolism of brain mitochondrial lipids was three times slower than that of the liver. 3. Individual lipids of brain mitochondria and myelin were also separated and their metabolism was studied. 4. All myelin lipids examined undergo very slow turnover. Two pools of brain mitochondrial lipid were identified. The slowly metabolized lipids were cholesterol, cardiolipin plus phosphatidic acid and possibly sphingomyelin; the remaining phosphatides underwent more rapid turnover. 5. The possible significance of these results is discussed.

It is now recognized that lipid together with protein forms an essential part of the mitochondrial membrane (Green & Fleischer, 1964). The precise role of this lipid has yet to be fully defined, either in the extent of its participation in the structure or in the more dynamic aspects of mitochondrial membrane function. Thus phospholipids are known to participate in certain mitochondrial enzyme systems and net lipid synthesis may also occur in the mitochondrion. Since it is probable that structural constituents are metabolically more stable than non-structural, long-term isotope experiments may be used to study the particular role of lipids in the mitochondrial membrane (Khan & Wilson, 1965; Davison & Gregson, 1966). In this paper such techniques have been utilized in single- or double-labelling experiments on newborn and young rats. The metabolism of each individual lipid of the brain mitochondrial membrane has been studied for a period of months to determine whether or not all the lipids are metabolically homogeneous, as appears to be the case for most of the myelin lipid (Cuzner, Davison & Gregson, 1965a). The turnover of brain mitochondrial lipids has also been compared with that of myelin and other subcellular structures. In this paper turnover has been taken as the rate of loss of radioactivity in relation to time. Since labelling varies from one lipid to another and even from one part of a lipid molecule to another, the turnover rate has been used only as a possible index of the behaviour of the mitochondrial membrane structure. A preliminary account of some of this work has been reported (Davison, 1966).

* Present address: Department of Physiological Chemistry, The Johns Hopkins University, Baltimore 5, Md., U.S.A.

METHODS

Animals. In the first experiment one litter of eight 17-day-old Wistar rats were given $10\mu\text{C}$ of $[1-^{14}\text{C}]$ acetate in 0.9% (w/v) sodium chloride solution by intraperitoneal injection. In the second experiment ten rats (two litters) were given 0.5 ml. of tritiated water ($20\mu\text{C}/\text{ml.}$) containing 0.9% (w/v) sodium chloride. Animals were injected intraperitoneally at 9 and 13 days *post partum*. When the rats were 53 days old they were injected with 0.5 ml. of 0.9% (w/v) sodium chloride solution containing $20\mu\text{C}$ of $[1-^{14}\text{C}]$ acetate. Rats were killed at intervals after injection.

Subcellular fractionation. Myelin and subcellular fractions were prepared by techniques similar to those described by Cuzner, Davison & Gregson (1965b); liver mitochondria were prepared in 0.32 M-sucrose solution containing 1 mM-EDTA but sedimented at 10500g for 15 min. after nuclei and cell debris had previously been removed by centrifuging at 900g for 10 min.

Method A. The following procedure was used in Expt. 1 only for the isolation of mitochondria from animals injected with $[1-^{14}\text{C}]$ acetate, as described above. The crude mitochondrial pellet from one rat brain was resuspended in a small volume of 0.32 M-sucrose containing 1 mM-EDTA. The suspension was distributed over four tubes containing 1.2 ml. of 0.8 M-sucrose and the whole centrifuged at 40000g for 60 min. in a swing-out head (MSE Superspeed 25, $6\times 3\text{ ml.}$). A loose superficial layer, containing mainly synaptic endings, was removed from the pellet formed at the bottom of the tube and the firmly packed material was resuspended in 0.32 M-sucrose to 10 ml. A portion (1 ml.) of suspension was removed for analysis and radioactivity determination. Deoxycholic acid (1 ml. of 3%, w/v, dissolved in 2.5 M-tris buffer, pH 7.4; Ulrich, 1960) was mixed with the remaining 9 ml. of mitochondrial suspension. After standing at 0° for 20 min. the preparation was centrifuged at 40000g for 90 min. with the swing-out head.

Method B. The following procedure was used in Expt. 2 for the isolation of mitochondria from animals doubly labelled, as described above. The crude mitochondrial pellet from rat brain was resuspended in 4 ml. of 0.8 M-

sucrose solution and 1 ml. of suspension layered over 1.2 ml. of 1.2M-sucrose solution. Approx. 0.5 ml. of 0.32M-sucrose-1 mM-EDTA was added to the top of each tube to facilitate removal of myelin after sedimentation. The tubes were centrifuged at 30500g for 60 min. to give a firmly packed pellet at the bottom of each tube.

Sample extraction and analysis. Tissue preparations were freeze-dried and extracted with 19 vol. of chloroform-methanol (2:1, v/v) plus 1 vol. of water, and proteolipid protein was removed from the filtered extract by using the method of Webster & Folch (1961). The extracts were dried under nitrogen, kept under vacuum overnight and weighed to give the lipid content.

Lipid samples were run on thin-layer chromatoplates, and the radioactive lipids quantitatively eluted, as described by Davison & Graham-Wolfaard (1964). Myelin lipid samples were developed in chloroform-methanol-aq. 32.5% ammonia (17:7:1, by vol.).

Mitochondrial lipid samples were developed in chloroform-methanol-aq. 20.3% ammonia (17:7:1, by vol.) to give effective separation of ethanolamine phospholipid, cardiolipin plus phosphatidic acid and cerebroside (M. L. Cuzner & A. N. Davison, unpublished work). Evidence for the quantitative reliability of cerebroside determination by thin-layer chromatography has been published (Davison & Graham-Wolfaard, 1964) and this work has since been confirmed. The validity of the thin-layer separation and analysis of cerebroside content of lipid extracts has been checked as follows. Purified cerebroside markers have been chromatographed on thin-layer plates alongside total lipid extracts and analysed by the same method. Galactose and sphingosine assays gave identical results with 95% recovery of the purified sample. Specific

sensitive chromatographic sprays, e.g. orcinol, show that the two cerebroside are clearly separated from all other lipids.

The individual radioactive lipids were dried in a stream of nitrogen or air, transferred to glass counting vials with 4 × 2 ml. of scintillator (0.3%, w/v, 2,5-diphenyloxazole in xylene) and counted in a Tri-Carb automatic scintillation counter, by using appropriate settings. Corrections were applied for sample quenching. The same samples, after satisfactory counting, were dried and analysed for lipid phosphorus, for sphingosine or for cholesterol (Davison & Graham-Wolfaard, 1964; Cuzner *et al.* 1965b). It was found that the presence of the scintillator did not interfere with these analytical determinations.

RESULTS

The first part of this work has been concerned with establishing a suitable technique for studying the turnover of brain mitochondrial lipid. Experiments with ³⁵S-labelled sulpholipids in intact rats had suggested that, apart from a few days *post partum*, metabolism of mitochondrial sulphatide was similar throughout life (Davison & Gregson, 1966). Since it was also intended to compare myelin with mitochondrial metabolism, actively myelinating rats were chosen for investigation. Thus 17-day-old rats were first injected with [1-¹⁴C]acetate and animals killed at intervals thereafter. Fractions were prepared from brain suspensions by method A, and the mitochondria

Table 1. *Chemical composition of rat-brain and liver mitochondrial fractions*

Mitochondrial fractions were prepared as described in the text. Fractions prepared by method A were further treated with deoxycholate (DOC) as described by Ulrich (1960) and the insoluble membrane was sedimented at 40000g for 90 min. Whole organelle composition of rat brain was that of mitochondria prepared by method B, and that for the liver was by method A. Lipid was extracted after freeze-drying. Full details of methods of analysis and extraction are as described in the text. Results are recorded as mg. of protein recovered/g. wet wt. of tissue of each organ with s.d. and number of observations in parenthesis. Lipid compositions are recorded as moles/100 moles of total lipid: five samples were analysed for brain and single samples for liver.

	Brain mitochondria			Liver mitochondria	
	Whole organelle	DOC-insoluble residue	DOC-soluble fraction	Whole organelle	DOC-insoluble residue
Recovered protein (mg./g. wet wt. of tissue)	10.22 ± 1.83 (8)	7.39 ± 3.32 (7)	—	49.36 ± 6.9 (6)	9.01 ± 4.58 (6)
Mean lipid composition (moles/100 moles)					
Cholesterol	25.3	30.2	34.6	11	5.3
Cerebroside	0.14	6.1	—	—	—
Cardiolipin	5.8	—	—	4.9	—
Phosphatidylserine	4.7	3.9	9.3	4.9	8.5
Phosphatidylinositol	3.7	1.9		7	
Sphingomyelin	1.4	3.6	4.6		4.1
Lecithin	28.3	30.6	25.5	41	37
Phosphatidylethanolamine	30.3	23.7	20.0	29	45.3*
Total phospholipid	74.2	63.7	—	89	94.9

* Includes cardiolipin.

separated into deoxycholate-soluble and -insoluble fractions. The deoxycholate-insoluble material from brain mitochondria was found by electron microscopy to consist of apparently homogeneous membrane fragments. Moreover, both the deoxycholate-soluble and -insoluble mitochondrial fractions had a similar lipid composition to that of the whole organelle (Table 1). No difference was detected in the turnover characteristics of whole mitochondrial lipid, compared with that of the deoxycholate-soluble and -insoluble fractions (Fig. 1).

Since no striking difference was seen in the behaviour and composition of the whole mitochondrial lipid compared with a separated membrane fraction, the deoxycholate treatment was not repeated in the second experiment. Attention was instead directed to preparing homogeneous mitochondrial fractions, for contamination by synaptic endings and by traces of lipid-rich myelin fragments has been reported (Petrushka & Giuditta, 1959; Aldridge & Johnson, 1959; Whittaker, 1959). To assess such possible contamination use was made of the differences in the long-term rate of metabolism of myelin constituents compared with

those of mitochondria (Davison & Gregson, 1966). For this purpose tritiated water was given to the rats during the period of maximum myelination and 40 days later the now young adult animals were injected with $[1-^{14}\text{C}]\text{acetate}$. Rats were killed at intervals up to 82 days thereafter. Brain suspensions were prepared and subcellular fractionation procedures, including a density-gradient separation, were as described under method B.

Turnover of whole lipids from brain subcellular fractions. The metabolism of lipids from various subcellular brain fractions has been studied by using the double-labelling technique. The results for turnover of whole-brain lipid show a relatively long half-life for both ^3H - and ^{14}C -labelled lipid. In most fractions turnover rates of both ^3H - and ^{14}C -labelled lipid were alike, with little evidence of biphasic decay (Khan & Wilson, 1965). This was in contrast with the labelled myelin fraction, where the very slow turnover was comparable with that found in many earlier experiments (Davison, 1964; Cuzner *et al.* 1965a). Results for the turnover of whole mitochondrial lipid labelled with both tritium and ^{14}C were not dissimilar (Fig. 1 and Table 2), the mean half-life being about 30 days. These long-term decay curves showed no obvious biphasic character (Khan & Wilson, 1965), or persistence of radioactivity as might have been expected if the samples had been significantly contaminated with myelin (Davison & Gregson, 1962). Turnover of brain mitochondrial lipid was considerably slower than that found for ^{14}C -labelled liver mitochondrial lipid (Fig. 1).

Metabolism of individual mitochondrial and myelin lipids of rat brain. Although the turnover rate of rat-brain whole mitochondrial lipids appeared to be uniform (Fig. 1), some differences in the metabolism of individual lipids was noted, after separation on thin-layer chromatograms. In both experiments there was little loss of incorporated ^{14}C or ^3H from cholesterol, cardiolipin + phosphatidic acid and possibly cerebroside and sphingomyelin (Table 3), whereas all other mitochondrial phosphatides underwent turnover (Fig. 2). No clear differences were noted in results obtained after tritium labelling early in life compared with those after ^{14}C labelling in the same animals 36 days later. In contrast, examination of the myelin fraction from rats doubly labelled with ^3H and ^{14}C showed the general metabolic stability of all the individual lipids (Table 4).

Homogeneity of the mitochondrial fraction. Mitochondria, prepared by method B, were layered on a Ficoll density gradient and centrifuged at $100\,000g$ for 30 min. in a Christ refrigerated centrifuge Omega II (Tanaka & Abood, 1963). The mitochondria collected as a pellet at the bottom of the tube. A barely visible white layer near the top

Fig. 1. Turnover of rat-brain and liver mitochondrial whole lipid. Results show the radioactivity remaining in whole-brain mitochondrial lipid at various times after injection with isotopic precursors. In Expt. 1 17-day-old rats were injected with $[1-^{14}\text{C}]\text{acetate}$ and lipid was extracted from isolated whole mitochondria (□) or from deoxycholate (DOC)-treated membrane fractions (●). Results for the second experiment in which tritium-treated rats were given $[1-^{14}\text{C}]\text{acetate}$ when 53 days old are shown as tritium radioactivity (△) and ^{14}C (○). Details of extraction, counting and DOC treatments are given in the text. The best straight line has been calculated by using the level of maximum incorporated radioactivity as 100. For the liver the calculated best straight line only is shown as a broken line using the results of Fletcher & Sanadi (1961). The calculated mean half-life of brain mitochondrial lipid is $t_{0.5} = 31.6$ days and for the liver mitochondrial lipid $t_{0.5} = 10.6$ days (Fletcher & Sanadi, 1961).

Table 2. Turnover and distribution of radioactivity in the lipids of brain subcellular fractions

Rats previously injected with tritiated water and with [1- ¹⁴ C]acetate were killed at intervals as described for Expt. 2. Radioactivity of total lipid for the whole of the various subcellular fractions was determined (see the Methods section) and the proportion of the whole sample used is shown below. Radioactivity was determined by counting all samples for 10 min. Counts were about ten times background except for determinations on the mitochondrial fraction, which were counted for longer periods to an error of less than 10% (s.e.m.). Half-lives have been calculated from the regression coefficient for each line. Each result is obtained from single animals except that for 82 days two brains were pooled. The regression coefficient (<i>b</i>), the correlation coefficient (<i>r</i>) and significance of the slope (<i>P</i>) have been calculated.																
Subcellular fraction	Isotope	Proportion of fraction counted (%)	Time (days after injection of ¹⁴ C).....										<i>b</i> (ln mμc/day)	<i>r</i>	<i>P</i> for <i>b</i> = 0	Half-life (days)
			Brain wt. (g.)													
Whole brain	³ H	8	25.25	15.90	18.30	13.69	12.03	9.14	13.15	9.61	−0.0089	−0.747	0.025−0.0125	77.2		
	¹⁴ C		1.49	1.06	1.55	1.60	0.828	0.78	0.81	0.63	−0.0104	−0.824	0.01−0.005	66.5		
Nuclei	³ H	84	0.76	0.65	0.72	0.752	0.86	0.62	0.51	0.39	−0.0078	−0.642	0.05−0.025	88.4		
	¹⁴ C		0.050	0.075	0.074	0.033	0.067	0.064	0.035	0.022	−0.0118	−0.727	0.025−0.0125	58.4		
Synaptic endings	³ H	42	2.51	3.93	2.02	1.35	2.97	1.0	2.37	1.14	−0.0093	−0.542	0.1−0.05	74.4		
	¹⁴ C		0.237	0.329	0.264	0.244	0.249	0.113	0.206	0.109	−0.0115	−0.784	0.01−0.005	60.4		
Mitochondria	³ H	8.5	0.63	0.35	0.48	0.37	0.41	0.23	0.34	0.14	−0.0140	−0.855	0.005−0.0025	49.6		
	¹⁴ C		0.146	0.074	0.091	0.088	0.051	0.045	0.032	0.017	−0.0233	−0.958	0.0025−0.0005	29.7		
Microsomes	³ H	84	3.27	1.68	2.20	1.67	1.37	1.04	1.11	0.83	−0.0135	−0.858	0.005−0.0025	51.1		
	¹⁴ C		0.274	0.149	0.224	0.276	0.133	0.116	0.090	0.067	−0.0162	−0.870	0.0025−0.0005	42.7		
Supernatant	³ H	16	3.77	4.05	3.26	1.44	2.03	1.35	0.97	0.64	−0.0222	−0.928	< 0.0005	31.2		
	¹⁴ C		0.391	0.414	0.362	0.145	0.173	0.142	0.092	0.051	−0.0253	−0.942	< 0.0005	27.4		
Myelin	³ H	8	8.30	6.25	8.33	8.27	4.97	4.90	5.17	4.70	−0.0070	−0.743	0.025−0.0125	99.3		
	¹⁴ C		0.190	0.228	0.268	0.296	0.226	0.298	0.319	0.265	+ 0.0019	+ 0.343	0.2−0.15	372		

Table 3. *Turnover and distribution of radioactivity in individual lipids from brain mitochondria*

Rats previously injected with [$1\text{-}^{14}\text{C}$]acetate alone (Expt. 1) and with tritiated water followed by [$1\text{-}^{14}\text{C}$]acetate (Expt. 2) were killed at intervals as described in the Methods section. Results for the radioactivity present in individual lipids separated by thin-layer chromatography from 88.5% of the whole-brain mitochondrial fraction are shown. The regression coefficient (b), the correlation coefficient (r), the half-life and significance of slope (P) have been calculated where appropriate. The standard error of sample counts has been determined: $\text{s.e.m.} = \sqrt{[2B + S]/t}$, where B is total background count, S is total sample count, t is time, background and sample counts being counted for 100 min.

Time (days after ¹⁴ C injection).....															b (ln mμC/ day)	r	P for b = 0	Half- life (days)
Expt. no.	Lipid fraction	Repre- sentative counting error (s.e.m.)																
		(mμC/whole brain equivalent)																
1 2	Cholesterol	¹⁴ C	3.11	3.99	2.66	2.84	0.155	3.52	0.152	2.31	0.159	2.28	0.133	—	0.0103	−0.690	0.1	Not signi- ficant
		³ H	below 5%	0.177	0.182	0.142	0.142	0.0107	0.0108	0.0108	0.0108	0.0107	0.013	0.0129	+0.0044	+0.904	0.001	
1 2	Phosphatidylethanol- amine	¹⁴ C	0.0108	0.0108	0.0108	0.0107	0.0134	0.012	0.012	0.77	0.013	1.04	0.073	0.073	−0.0320	−0.895	0.05−0.01	22
		³ H	2.91	2.95	2.92	2.02	0.138	1.99	0.125	0.125	0.096	0.096	1.04	0.073	−0.0205	−0.999	0.001	34
1 2	Lecithin	¹⁴ C	below 5%	0.194	0.205	0.177	0.0278	0.042	0.012	0.012	0.0158	0.0158	0.0116	0.0116	−0.0197	−0.888	0.05−0.01	35
		³ H	4.5	4.55	1.82	3.26	0.075	1.71	0.058	0.520	0.046	0.358	0.050	0.050	−0.0606	−0.987	0.01−0.001	11
1 2	Cardiolipin + phosphatidic acid	¹⁴ C	below 10%	0.154	0.146	0.125	0.0317	0.039	0.0134	0.018	0.018	0.423	0.0097	0.0097	−0.0367	−0.984	0.001	19
		³ H	0.061	0.0822	0.079	0.246	0.030	0.446	0.026	—	0.0194	0.0245	0.0245	0.0245	—	Not calc.	0.05−0.01	23
1 2	Phosphatidylserine	¹⁴ C*	10−20%	0.270	0.32	0.293	0.246	0.0089	0.0049	0.0049	0.0065	0.195	0.0124	0.0124	−0.0094	−0.751	0.05	73.7
		³ H	below 10%	0.038	0.036	0.023	0.035	0.0099	0.0049	0.0049	0.0065	0.195	0.0124	0.0124	−0.0053	−0.274	0.1	
1 2	Phosphoinositide	¹⁴ C	10−20%	0.222	0.32	0.293	0.246	0.015	—	0.0154	0.0082	0.195	0.0124	0.0124	−0.0257	−0.853	0.05−0.01	27
		³ H	below 10%	0.038	0.036	0.021	0.021	0.0061	0.0061	0.0030	0.0030	0.0030	0.0021	0.0021	−0.0251	−0.946	0.05−0.01	
1 2	Sphingomyelin	¹⁴ C	>20%	0.153	0.168	0.088	0.100	0.107	0.107	—	0.0045	—	0.0068	0.0068	−0.024	−0.837	0.1−0.05	29
		³ H	10−20%	—	0.0152	0.0176	0.0176	0.0028	0.0031	0.0031	0.0022	0.130	0.0011	0.0011	−0.021	−0.872	0.1−0.05	34
1 2	Cerebroside	¹⁴ C	>20%	0.468	0.056	0.076	0.121	0.281	0.281	0.0046	0.0082	0.130	0.0053	0.0053	—	Not calc.	>0.1	
		³ H	10−20%	—	0.011	0.0031	0.0031	0.0023	0.0023	0.0024	0.0026	0.0026	0.0002	0.0002	+0.0070	+0.064	>0.1	
1 2	Cerebroside	¹⁴ C	>20%	—	—	—	—	—	—	—	—	—	—	—	—	Not calc.	>0.1	
		³ H	10−20%	0.023	0.0138	0.0154	0.0154	0.0152	0.026	0.0010	0.0010	0.0104	0.0079	0.0079	−0.0145	−0.643	>0.1	
1 2	Cerebroside	¹⁴ C	>20%	0.0021	0.0017	—	—	0.0006	0.0006	0.0006	0.0022	0.0022	0.0002	0.0002	—	Not calc.	>0.1	
		³ H	10−20%	—	—	—	—	—	—	—	—	—	—	—	—	Not calc.	>0.1	

* Includes cerebroside in Expt. 1 only.

of the gradient was shown by electron microscopy to consist largely of myelin. Lipid analysis was consistent with this observation (Table 5). To have a quantitative assessment of possible myelin contamination, the separation on a Ficoll density gradient was repeated on the brains of four animals

pre-treated 100 days earlier with $[1-^{14}\text{C}]\text{glucose}$. Even after this long period, only 12% of the mitochondrial total lipid radioactivity could be accounted for by the contaminating myelin layer. This was equivalent to less than 1% of the myelin total lipid radioactivity. Again, chemical analysis was consistent with this conclusion, for almost all the typical myelin lipid, cerebroside, present in unpurified mitochondria, could be accounted for in the myelin-rich fraction, separated on the Ficoll density gradient and less than 6% of total brain mitochondrial cholesterol was found in this fraction. Only very small amounts of cerebroside were present in the purified mitochondrial pellet. Recalculation of turnover rates, with respect to the myelin contamination, made no significant difference to values for half-lives.

DISCUSSION

Fletcher & Sanadi (1961) have presented convincing evidence that turnover of total liver mitochondrial lipid is representative of the turnover of the whole organelle structure. Experiments reported in this paper (Table 2) suggest that the same applied to total brain mitochondrial lipid. Thus membrane fragments obtained from deoxycholate-treated mitochondria have the same lipid composition as the intact organelle, suggesting that most of the lipid is concentrated in the membrane. In addition no difference was noted between the metabolic behaviour of the lipids of fragmented and whole mitochondria (Fig. 1). For these reasons, in the second experiment metabolism of the whole

Fig. 2. Turnover of rat-brain lipids. Results show the radioactivity remaining in representative lipids separated from the deoxycholate-treated rat brain mitochondrial preparation at intervals after injection of $[1-^{14}\text{C}]\text{acetate}$ into 17-day-old rats (Expt. 1). Radioactivity is expressed as counts/whole organ/100sec. Cholesterol (●) and lecithin (Δ) have been chosen as representative mitochondrial lipids. Full details of experimental procedures are given in the text under Expt. 1 above.

Table 4. *Turnover and distribution of radioactivity in the individual lipids of myelin from rat brain*

Rats previously injected with tritiated water and with $[1-^{14}\text{C}]\text{acetate}$ were killed at intervals as described for Expt. 2. Radioactivity of individual lipids separated by thin-layer chromatography is shown.

Lipid fraction	Time (days after injection of ^{14}C)..... Isotope	Radioactivity ($\text{m}\mu\text{C}/\text{whole brain}$)					Half-life where regression coefficient significant (days)
		1	3	8	25	51	
Total myelin lipid	^3H	9.1	11.1	12.1	8.2	7.5	No significant turnover
	^{14}C	0.187	0.333	0.529	0.413	0.376	—
Cholesterol	^3H	2.80	2.90	2.57	2.58	2.16	136
	^{14}C	0.069	0.095	0.133	0.145	0.150	—
Cerebroside	^3H	0.505	0.400	0.774	0.383	0.181	No significant turnover
	^{14}C	0.0048	0.0118	0.0197	0.0133	0.0070	—
Lecithin	^3H	1.515	1.165	0.580	1.07	0.560	No significant turnover
	^{14}C	0.071	0.056	0.033	0.086	0.048	—
Phosphatidylserine	^3H	0.380	0.358	0.445	0.301	0.227	62
	^{14}C	0.013	0.0114	0.029	0.013	0.0203	—
Phosphatidylethanolamine	^3H	1.234	2.90	1.39	1.94	1.90	No significant turnover
	^{14}C	0.032	0.058	0.053	0.051	0.070	—
Phosphoinositide + sphingomyelin	^3H	0.100	0.236	0.234	0.094	0.049	No significant turnover
	^{14}C	0.0035	0.0066	0.014	0.0036	—	—

Table 5. *Analysis of brain mitochondria after Ficoll-density-gradient separation*

Three separate samples of adult brain mitochondria were prepared by method B from four, two and two rats respectively. The mitochondria were layered on a Ficoll density gradient as described by Tanaka & Abood (1963). After separation, samples were taken for electron microscopy and the rest was analysed for lipid content as described in the Methods section. Less than 6% of the total mitochondrial cholesterol was present in the contaminating myelin layer.

Layer	Electron microscopy	Lipid molar proportions		
		Cholesterol	Phospholipid	Cerebroside
Thin white layer at top of gradient	Predominantly myelin	1	0.93	0.17
Buff-coloured loosely packed pellet	Mitochondria, no myelin	1	3.2	0.04
Buff-coloured densely packed pellet	Mitochondria, no myelin	1	2.7	0.04

mitochondrial lipid only has been studied. In experiments in which the metabolism of protein, cytochrome *c* and lipid were studied, Fletcher & Sanadi (1961) reported turnover rates equivalent to a half-life of 10.6 days. Metabolism of whole rat-brain mitochondrial lipid has been found to be about three times slower than that of the liver (Table 2 and Khan & Wilson, 1965). However, mitosis is restricted in the brain (Leblond, Messier & Kopriwa, 1959; Messier & Leblond, 1960) compared with other organs and this could perhaps be reflected in a differing metabolism of brain sub-cellular particles and their constituent lipids. Earlier work on the metabolism of protein and individual lipids of the myelin sheath (Davison, 1964; Cuzner *et al.* 1965a) showed that these constituents all underwent the same slow turnover (see Table 4). In view of these results the metabolism of individual brain mitochondrial lipids was investigated. It was found that inositol, serine, ethanolamine and choline phosphatides turned over with an average half-life of about 26 days. During the course of the experiment little or no turnover was noted in the labelled cholesterol, sphingomyelin, cardiolipin plus phosphatidic acid and cerebroside fractions (Table 3). It should be noted that the cardiolipin and phosphatidic acid fraction contains predominantly cardiolipin for only traces of phosphatidic acid have been found in brain mitochondria (Eichberg, Whittaker & Dawson, 1964). In the young animals injected with either [^{14}C]-acetate or tritiated water about a third of the total radioactivity incorporated into mitochondrial lipid was found in the stable lipid at the beginning of the experiment. However, in 53-day-old rats less than a fifth of the radioactivity was present in this same fraction 1 day after giving [^{14}C]acetate. These combined results on brain mitochondrial lipids clearly differ from those obtained in studies of myelin metabolism (Davison, 1964; Cuzner *et al.* 1965a) and those for liver mitochondria.

There are several possible explanations for the differences found in metabolism of the individual

brain mitochondrial lipids. Khan & Wilson (1965) found persistent radioactivity in brain mitochondrial ethanol extracts prepared from rats given tritiated water during gestation and up to 26 days *post partum*. In this experiment, mitochondria prepared from the brains of two rats more than 200 days after tritium withdrawal were resedimented in a sucrose-density gradient (De Robertis, Pellegrino de Iraldi, Rodriguez de Iraldi & Salganicoff, 1962). These purified mitochondria were found to contain no detectable radioactivity. It was therefore concluded by Khan & Wilson (1965) that this residual radioactivity represented a non-dynamic contaminant. In the present work the possibility that the mitochondrial pellet was contaminated with small myelin fragments was also considered. Lipid analysis showed that cerebroside, a typical myelin constituent, was present in small amounts in brain mitochondrial extracts. The mitochondrial preparation was found to contain small amounts of myelin, for on further fractionation of the mitochondria on a Ficoll density gradient (Tanaka & Abood, 1963) mitochondria containing only traces of cerebroside were isolated with a contaminating cerebroside-rich myelin layer appearing at the top of the gradient. These results are consistent with those of Smith & Eng (1965), who submitted rat-brain mitochondria to repeated layering and repurification (Table 6). Differences between our results and those of other authors (Table 6) may be due to species differences (Eichberg *et al.* 1964), to the presence of various amounts of myelin and other contaminants in the mitochondrial fractions or to different analytical methods employed.

Metabolic observations support the view that only small amounts of contaminating myelin were present in our mitochondria, for ethanolamine phospholipid undergoes fast turnover in the rat-brain mitochondrial preparations yet in myelin, where it is a major component, it is metabolically stable (Cuzner *et al.* 1965a); on the other hand cardiolipin, a typical mitochondrial lipid, is unex-

Table 6. *Composition of rat-brain mitochondrial lipid*

Composition of rat-brain mitochondrial lipid as reported by various workers by using different fractionation and analytical methods. Results are shown as molar ratios with cholesterol equalling one.

Lipid molar proportions			Treatment	Reference
Cerebroside and sulphatide	Cholesterol	Phospholipid		
0.15	1	2.43	Repeated layering and repurification	Smith & Eng (1965)
0.25	1	3	One layering on sucrose density gradient	Seminario, Hren & Gomez (1964)
—	1	2.01	Myelin must be included in the fraction	Biran & Bartley (1961)
1.66	1	7.2	Reprecipitation and washing	Løvtrup & Svennerholm (1963)
0.2	1	2.1	Before purification	This paper
0.06	1	2.93	After purification	This paper
0.22*	1	7	Layering only	Eichberg <i>et al.</i> (1964)

* Guinea pig analysed (not rat).

pectedly metabolically stable. Two other hypotheses were considered. First, it seemed possible that retention of radioactively labelled lipid in the rat-brain mitochondria was due to reincorporation of some of the labelled lipid. Certain evidence is consistent with this hypothesis. Catabolism of cerebroside and particularly cholesterol is known to be restricted within the nervous system (Davison, 1965; Dobbing, 1963) and phospholipids such as lecithin undergo dynamic metabolism (Ansell & Dohmen, 1959; Dawson & Richter, 1950). Although ³⁵S-labelled mitochondrial sulphatide has been found to undergo turnover with a half-life of about 39 days (Davison & Gregson, 1966), this can be correlated with the presence of a brain sulphatidase (McKhann, Levy & Ho, 1965). A further explanation is that the metabolically more stable lipids are possibly part of the basic unit membrane of the organelle whereas the other lipids are non-structural and can undergo exchange and catabolism. Such a concept would be consistent with the observations of Luck (1963), which suggest that yeast mitochondrial membrane remains intact during mitochondrial replication. This same hypothesis may be related to the stability of the cell population of the central nervous system compared with other organs. Recent work on the metabolism of mitochondrial lipids from rat liver also shows, contrary to the earlier conclusions of Fletcher & Sanadi (1961), that there are two lipid pools undergoing different rates of turnover, the pool with the longer half-life probably representing replacement of the mitochondrial structure (Gurr, Prottey & Hawthorne, 1966; Bailey, Taylor & Bartley, 1966).

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