Chemical Studies of Peripheral Nerve During Wallerian Degeneration

1. LIPIDS

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When a peripheral nerve is cut, that portion of the nerve distal to the point of section soon loses its ability to transmit a nerve impulse. This is associated with a series of familiar histological changes known as Wallerian degeneration. Both histologists and histochemists have studied degenerating nerve in great detail, but in few instances have the techniques of chemistry been employed. Notable exceptions are the classical publications of Noll (1899), who observed that degenerating horse and dog nerves contained less 'protagon', and Mott & Halliburton (1901*a*, *b*), who reported the complete disappearance of phosphorus from degenerating cat nerves. These results, although of great interest, are of limited value because of the technical methods employed; usually a large sample of nerve tissue was needed and only one constituent of the nerve could be estimated from each sample. More recently, May (1930) has reported changes in the distribution of phosphorus and Abercrombie & Johnson (1946*b*) have described changes in the distribution of nitrogen, including collagen nitrogen, in degenerating rabbit nerves.

As the nerve degenerates, the lipid-containing myelin sheath that surrounds the axon of each individual nerve fibre at first fragments and later is completely destroyed. Although the principal constituent of this sheath, 'myelin', is usually referred to as though it were a chemical entity, chemists have for a long time suspected it to be a mixture of substances. Recently, Johnson, McNabb & Rossiter (1948*a*, *b*; 1949*a*) have produced evidence that free cholesterol and the two sphingosine-containing lipids or sphingolipids (cerebroside and sphingomyelin), rather than lecithin or kephalin, are the principal lipid components of the myelin sheath. They have called these lipids, i.e. free cholesterol, cerebroside and sphingomyelin, the myelin lipids.

For references to the extensive literature on the histological changes that occur during Wallerian degeneration the reader is referred to the publications of Ramon y Cajal (1928), Nageotte (1932), Weddell & Glees (1941), Young (1942), Holmes & Young (1942) and Weiss (1944). Since destruction of the myelin sheath is such an outstanding feature, it seemed of interest to investigate the changes in the concentration of the myelin lipids in a degenerating nerve. Micromethods are now available that permit the determination of these and other lipids in small samples of nerve. A preliminary account of these experiments has already appeared (Johnson, McNabb & Rossiter, 1949*b*).

METHODS

The right sciatic nerve of 30 cats was cut at the level of the greater trochanter of the femur. The proximal stump was retracted and sutured to the overlying muscle. By separating the proximal and distal stumps in this way the possibility of regeneration was minimized. None of the animals showed either functional or post-mortem evidence of regeneration. The operation was performed with full aseptic precautions under nembutal anaesthesia. No attempt was made to control the age, weight, or sex of the animals. After periods of time varying from 4 to 96 days the animals were killed and the distal degenerating segment of nerve removed. At the same time a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue and the lipids extracted with ethanol and ether as described previously (Johnson *et al.* 1948*a*). Samples of the ethanol-ether extract were placed in a 60° water bath and evaporated just to dryness under reduced pressure and in an atmosphere of N_2. The lipids were extracted with light petroleum (b.p. 40–60°) and the phospholipins precipitated with acetone and $MgCl_2$. The precipitate was washed with acetone and then dissolved in a 1:1 methanol-ether mixture.

The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin, total fatty acid and neutral fat was determined in each nerve. From these figures the concentration of ester cholesterol, sphingomyelin, kephalin, essential lipid (i.e. all non-triglyceride lipids), myelin lipid, and total lipid was calculated. All analyses were done in duplicate.

Analytical procedures

The analytical methods for cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin and lecithin have been described by Johnson *et al.* (1948*a*).

Cerebroside (glycosphingoside) was estimated in a sample of the original ethanol-ether extract by the method of Brand & Sperry (1941) in which galactose, liberated by acid hydrolysis, is estimated by the procedure of Miller & Van Slyke (1936). In this method, ferrocyanide, formed from the reduction of ferricyanide by the galactose, is titrated with ceric sulphate. Cerebroside was calculated as galactose $\times 4{\cdot}5$.

Total and free cholesterol were estimated in the acetone-soluble fraction by the method of Schoenheimer & Sperry (1934), incorporating improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945).

Total phospholipin. Total P was determined in a sample of the methanol-ether solution of the acetone-precipitated phospholipin by the method of King (1932). Total phospholipin was calculated as lipid P $\times 25$.

Monoaminophospholipin (phosphoglyceride). A sample of the methanol-ether solution of the acetone-precipitated phospholipins was hydrolysed in N-KOH for 18 hr. at 37°. Monoaminophospholipin was estimated as the total acid-soluble P of the hydrolysate (Schmidt, Benotti, Hershman & Thannhauser, 1946).

Lecithin (phosphatidyl choline) was estimated as the acid-soluble choline, determined by the method of Glick (1944), in the monoaminophospholipin hydrolysate (Hack, 1947). Lecithin was calculated as choline chloride $\times 5{\cdot}56$.

Total fatty acid was determined in a sample of the original ethanol-ether extract by a modification of the Bloor oxidative method described by Boyd (1938). After saponification and acidification, the free fatty acid and cholesterol were extracted with light petroleum (b.p. 40–60°) and oxidized by a measured quantity of $K_2Cr_2O_7$, excess of which was titrated with thiosulphate. After correction had been made for the cholesterol present, the total fatty acid was calculated on the assumption that 1 mg. of fatty acid was oxidized by 3·61 ml. 0·100 N-$K_2Cr_2O_7$ (Boyd, 1938).

Neutral fat. An estimate of triglyceride was obtained by determining the glycerol in a portion of the phospholipin-free acetone solution by the method of Voris, Ellis & Maynard (1940). After saponification and acidification, the fatty acids and cholesterol were removed by extraction with light petroleum (b.p. 40–60°) and the glycerol allowed to react with a measured quantity of potassium periodate. Excess periodate was titrated with thiosulphate. The periodate used gave a measure of the glycerol in the sample. Neutral fat, based on an assumed mean molecular weight of 283 for the fatty acids, was calculated as glycerol $\times 9{\cdot}64$.

In addition, a value for neutral fat was derived from the figures for neutral-fat fatty acid, i.e. the difference between the total fatty acid and the sum of the fatty acids of cerebroside, cholesterol ester, lecithin, sphingomyelin and kephalin. In the absence of detailed information concerning the nature of the fatty acids of the lipids of nerve,* the figures were calculated on the basis of an assumed mean molecular weight of 368, that of lignoceric acid, for the fatty acids of the two sphingolipids, cerebroside and sphingomyelin, and an assumed mean molecular weight of 283 for the fatty acids of the phosphoglycerides and cholesterol ester. A molecular weight differing from the assumed mean for the fatty acids of either the sphingolipids or the phosphoglycerides would not appreciably alter the general conclusions. The neutral-fat fatty acids were, therefore, calculated as: total fatty acid (cerebroside $\times 0{\cdot}45$ + ester cholesterol $\times 0{\cdot}73$ + lecithin $\times 0{\cdot}70$ + sphingomyelin $\times 0{\cdot}44$ + kephalin $\times 0{\cdot}74$). Based on an assumed mean of 283 for the triglyceride fatty acids, the neutral fat was calculated from the neutral-fat fatty acid by multiplying by the factor 1·045.

Ester cholesterol was calculated as the difference between the total cholesterol and the free cholesterol.

Sphingomyelin (phosphosphingoside) was calculated as the difference between the total phospholipin and the monoaminophospholipin.

Kephalin, representing all the non-choline-containing monoaminophospholipins, was calculated as the difference between the monoaminophospholipin and lecithin.

Essential lipid, representing the non-triglyceride lipid, was the sum of cerebroside, total cholesterol and total phospholipin.

Myelin lipid was the sum of cerebroside, free cholesterol and sphingomyelin.

Total lipid was the sum of the essential lipid and neutral fat.

Specificity of analytical methods

The specificity of a micromethod, when it is usually impossible to characterize fully the substance to be estimated, is frequently far from absolute. Although we feel that, for the most part, we have estimated the substance for which each method was designed, it is important that possible deficiencies of the methods should not be forgotten. For instance, any substance, other than the known cerebrosides, that is soluble in the solvents used and from which reducing substances are released on acid hydrolysis, would be estimated as cerebroside. A similar qualification applies to the method for cholesterol. Although this method can now be described as standard, it is possible that small quantities of other digitonin-precipitable substances that give the Liebermann-Burchard colour reaction would be determined as cholesterol.

The method for total phospholipin is also standard, but that for monoaminophospholipin is less so. In this method known monoaminophospholipins, such as lecithin (phosphatidyl choline) and the kephalins (phosphatidyl ethanolamine, phosphatidyl serine and brain phosphoinositide), would be measured together with any other unknown easily hydrolysed phospholipins. If such substances contained choline in a form readily liberated on hydrolysis, they would be estimated as lecithin; if, as seems more likely, such unknown phospholipins contained no easily hydrolysed choline, they would be estimated as kephalin. In degenerating nerve, however, it is possible that choline-containing degradation products of either sphingomyelin or lecithin may be present. If such a substance were both soluble in light petroleum and insoluble in acetone, it would be estimated as lecithin. One possible degradation product of lecithin, β-glycerylphosphorylcholine, studied by Schmidt, Hershman & Thannhauser (1945), is insoluble in light petroleum, and so would not be present in the phospholipin extracts. Since sphingomyelin is measured as the difference between the total P and the readily hydrolysed P of the

* See, however, Chibnall, Piper & Williams (1936), who found that lignoceric acid from the brain cerebroside, kerasin, was a mixture of *n*-heneicosane-, *n*-tricosane- and *n*-pentacosane-1-carboxylic acids, whilst phrenosinic acid from the brain cerebroside, phrenosin, was a mixture of 2-hydroxy-*n*-heneicosane-, -*n*-tricosane- and -*n*-pentacosane-1-carboxylic acids.

lipid extract, unknown phospholipins, not easily hydrolysed, would be determined as sphingomyelin.

The method for total fatty acid is also not specific, and, in addition to cholesterol, for which correction is made, other non-saponifiable lipids may be measured. The sphingolipids are difficult to saponify and, even if saponification is complete, part of the liberated sphingosine may pass into the light petroleum together with the fatty acids and cholesterol. This may account for the difference in the figures for neutral fat calculated from neutral-fat fatty acid and those calculated from glycerol (some 30% for the control nerves). Also the glycerol of any degradation product of either triglyceride or phosphoglyceride that is soluble in light petroleum and not precipitated by acetone would be measured by the methods used.

Accuracy of analytical methods

The coefficient of variation of the overall procedure for each of the individual lipids was as follows: cerebroside ±3·5%, free cholesterol ±0·9%, total cholesterol ±0·9%, total phospholipin ±1·3%, monoaminophospholipin ±1·6%, lecithin ±5·1%, total fatty acid ±3·1%, and neutral fat ±3·1%. The accuracy of the methods for those lipids derived by difference would usually be less. Since the mean of duplicate estimations was always recorded, the probable error of each observation was less than the above figure.

Recording of results

For the first 32 days after the operation the wet weight of the degenerating nerve was greater than that of the same length of nerve from the control side. This presented the problem of how best to record the results. To express the results in terms of unit wet weight of the degenerating nerve would be misleading, while to express them in terms of unit dry weight of the degenerating nerve would also be unsatisfactory, for the degenerating nerve contained much less lipid than the normal nerve, and lipid accounts for a high percentage of the dry weight of nerve. Therefore we have expressed all results for degenerated nerve in terms of the wet weight of the same length of control nerve from the opposite side. This is equivalent to the fresh weight of the degenerating nerve before it was sectioned, i.e. at zero time.

RESULTS

Animals were killed at intervals of 4, 8, 16, 32, 64 and 96 days after the nerve section. Table 1 gives the mean and the standard error of the mean for the concentration of cerebroside, total, free and ester cholesterol, total phospholipin and essential lipid in the control and degenerated nerves. The table also gives the value of P obtained for each lipid in testing the significance of the difference between the means for the degenerated and control nerves.

The concentration of cerebroside, 2·4 mg./100 mg. for the control nerves, changed little during the first 8 days of degeneration and then decreased rapidly, reaching 0·6 mg./100 mg., or 25% of the total, by 32 days. The concentration of cerebroside then decreased more gradually, no measurable cerebroside remaining by 96 days.

Table 1. *Lipids of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve.)

	Control		Degenerated (4 days)			Degenerated (8 days)			Degenerated (16 days)		
No. of animals ...	30		5			7			5		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Cerebroside	2·40	±0·11	2·48	±0·32	>0·7	2·30	±0·23	>0·6	1·32	±0·12	<0·01
Total cholesterol	3·21	±0·05	3·22	±0·16	>0·9	3·09	±0·15	>0·3	2·66	±0·19	<0·01
Free cholesterol	3·19	±0·05	3·17	±0·16	>0·8	2·92	±0·13	<0·05	1·66	±0·06	<0·01
Ester cholesterol (total – free cholesterol)	0·02	±0·02	0·04	±0·02	>0·2	0·17	±0·04	<0·01	1·00	±0·17	<0·01
Total phospholipin	6·04	±0·13	6·12	±0·26	>0·8	6·00	±0·55	>0·9	2·98	±0·13	<0·01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11·63	±0·26	11·82	±0·56	>0·3	11·43	±0·87	>0·7	6·96	±0·29	<0·01

	Control		Degenerated (32 days)			Degenerated (64 days)			Degenerated (96 days)		
No. of animals ...	30		5			5			3		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Cerebroside	2·40	±0·11	0·60	±0·16	<0·01	0·48	±0·11	<0·01	0	±0	<0·01
Total cholesterol	3·21	±0·05	1·94	±0·19	<0·01	1·02	±0·05	<0·01	0·77	±0·05	<0·01
Free cholesterol	3·19	±0·05	1·15	±0·05	<0·01	0·54	±0·06	<0·01	0·29	±0·05	<0·01
Ester cholesterol (Total – free cholesterol)	0·02	±0·02	0·79	±0·35	<0·01	0·48	±0·07	<0·01	0·47	±0·08	<0·01
Total phospholipin	6·04	±0·13	1·30	±0·13	<0·01	0·74	±0·05	<0·01	0·60	±0	<0·01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11·63	±0·26	3·86	±0·26	<0·01	2·22	±0·14	<0·01	1·37	±0·04	<0·01

The concentration of total cholesterol, 3·21 mg./100 mg. in the control nerves, changed little during the first 8 days and then decreased steadily during the course of the degeneration, 0·77 mg./100 mg. still remaining after 96 days. On the other hand, free cholesterol, which accounted for almost all of the cholesterol of the control nerves, decreased to 2·92 mg./100 mg. ($P < 0·05$) after 8 days. From 8 to 32 days the concentration of free cholesterol fell rapidly, and thereafter more slowly, only 0·29 mg./100 mg. remaining after 96 days. There was virtually no ester cholesterol in the control nerves and during the period 0–8 days the concentration increased slowly, reaching 0·17 mg./100 mg. ($P < 0·01$) by the 8th day. The concentration of ester cholesterol then increased rapidly, reaching a maximum of 1·0 mg./100 mg. in 16 days. After 16 days the concentration decreased, but more slowly than the concentration of free cholesterol, so that by 96 days more of the cholesterol was in the ester form than in the free.

The concentration of total phospholipin, which was 6·04 mg./100 mg. for the control nerves, also changed little during the first 8 days. It then decreased rapidly between 8 and 32 days and thereafter more slowly, only 0·6 mg./100 mg., or 10 % of the total, remaining after 96 days. The concentration of essential lipid, representing the sum of the concentrations of cerebroside, total cholesterol and total phospholipin, was 11·63 mg./100 mg. in the control nerves. This decreased in a manner similar to the total phospholipin, only somewhat more slowly. By 96 days 1·37 mg./100 mg. essential lipid remained, or 12 % of the control value.

Table 2 gives the figures for the individual phospholipins, viz. lecithin, sphingomyelin and kephalin, and also those for monoaminophospholipin, total phospholipin and myelin lipid. The concentration of none of these substances changed appreciably during the first 8 days. Between 8 and 32 days the concentration of total phospholipin, sphingomyelin and myelin lipid decreased rapidly and after 32 days more slowly, 10, 7 and 6 % respectively remaining after 96 days. Kephalin, the concentration of which was 2·97 mg./100 mg. in the control nerves, disappeared very rapidly between 8 and 32 days, 0·28 mg./100 mg., or only 9 % of the total, remaining at the end of this time. Subsequently, the concentration of kephalin did not change greatly. Lecithin, which

Table 2. *Phospholipins of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve)

	Control		Degenerated (4 days)			Degenerated (8 days)			Degenerated (16 days)		
No. of animals ...	30		5			7			5		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total phospholipin	6·04	±0·13	6·12	±0·26	>0·8	6·00	±0·55	>0·9	2·98	±0·13	<0·01
Monoaminophospholipin	3·70	±0·14	4·08	±0·43	>0·2	3·84	±0·28	>0·6	1·60	±0·12	<0·01
Lecithin	0·73	±0·04	0·80	±0·05	>0·4	0·82	±0·06	>0·2	0·51	±0·10	<0·05
Sphingomyelin (Total phospholipin – monoaminophospholipin)	2·33	±0·12	2·00	±0·39	>0·3	2·19	±0·40	>0·6	1·36	±0·12	<0·01
Kephalin (Monoaminophospholipin – lecithin)	2·97	±0·13	3·32	±0·46	>0·3	3·01	±0·26	>0·8	1·12	±0·15	<0·01
Myelin lipid (Cerebroside + free cholesterol + sphingomyelin)	7·91	±0·21	7·72	±0·69	>0·7	7·43	±0·70	>0·3	4·34	±0·16	<0·01

	Control		Degenerated (32 days)			Degenerated (64 days)			Degenerated (96 days)		
No. of animals ...	30		5			5			3		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total phospholipin	6·04	±0·13	1·30	±0·13	<0·01	0·74	±0·05	<0·01	0·60	±0	<0·01
Monoaminophospholipin	3·70	±0·14	0·66	±0·09	<0·01	0·48	±0·04	<0·01	0·40	±0	<0·01
Lecithin	0·73	±0·04	0·36	±0·03	<0·01	0·24	±0·03	<0·01	0·18	±0·03	<0·01
Sphingomyelin (Total phospholipin – monoaminophospholipin)	2·33	±0·12	0·66	±0·13	<0·01	0·24	±0·07	<0·01	0·17	±0·04	<0·01
Kephalin (Monoaminophospholipin – lecithin)	2·97	±0·13	0·28	±0·08	<0·01	0·24	±0·02	<0·01	0·23	±0·04	<0·01
Myelin lipid (Cerebroside + free cholesterol + sphingomyelin)	7·91	±0·21	2·40	±0·27	<0·01	1·28	±0·17	<0·01	0·50	±0·06	<0·01

was present to the extent of only 0·73 mg./100 mg. in the control nerves, decreased less rapidly than either the total phospholipin or myelin lipid.

Table 3 gives the figures for total fatty acid, neutral fat calculated from glycerol, neutral fat calculated from neutral-fat fatty acid, and total lipid.

Table 3. *Fatty acids and neutral fat of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve. No. of animals is stated in parentheses under each result.)

	Control	Degenerated (4 days)			Degenerated (8 days)			Degenerated (16 days)		
	Mean S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Total fatty acid	14·54 ±0·50 (20)	10·30	±1·13 (3)	<0·01	11·26	±1·00 (5)	<0·01	10·48	±0·83 (4)	<0·01
Neutral fat (calculated from glycerol)	6·91 ±0·44 (19)	4·41	±0·76 (4)	<0·05	3·65	±0·27 (4)	<0·01	6·22	±0·88 (3)	>0·5
Neutral fat (calculated from neutral-fat fatty acid)	10·14 ±0·60 (20)	5·24	±1·00 (3)	<0·01	6·60	±0·59 (5)	<0·01	7·78	±0·94 (4)	>0·1
Total lipid (Essential lipid + neutral fat)	18·36 ±0·40 (19)	16·00	±1·11 (4)	<0·05	14·52	±1·65 (4)	<0·01	12·80	±1·09 (3)	<0·01

	Control	Degenerated (32 days)			Degenerated (64 days)			Degenerated (96 days)		
	Mean S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Total fatty acid	14·54 ±0·50 (20)	12·05	±2·86 (2)	>0·1	10·57	±0·54 (3)	<0·01	10·37	±2·33 (3)	<0·02
Neutral fat (calculated from glycerol)	6·91 ±0·44 (19)	7·49	±3·97 (2)	>0·7	9·58	±0·57 (3)	<0·05	6·61	±1·69 (2)	>0·8
Neutral fat (calculated from neutral-fat fatty acid)	10·14 ±0·60 (20)	11·02	±2·94 (2)	>0·6	9·85	±0·58 (3)	>0·8	10·10	±2·37 (3)	>0·9
Total lipid (Essential lipid + neutral fat)	18·36 ±0·40 (19)	11·35	±3·35 (2)	<0·01	11·87	±0·59 (3)	<0·01	7·95	±1·75 (2)	<0·01

The concentration of total fatty acid fell from 14·54 mg./100 mg. to 10·30 mg./100 mg. in 4 days ($P < 0·01$) and remained close to this figure throughout the course of the degeneration. For each of the periods studied the difference between the values for the degenerating and control nerves was statistically significant, except for the 32-day nerves where fatty-acid figures were available for two animals only. It is likely that, had the group been larger, this figure also would have been significant.

The concentration of neutral fat estimated as glycerol was 6·91 mg./100 mg. in the control nerves. This fell to 4·41 mg./100 mg. after 4 days ($P < 0·05$) and 3·65 mg./100 mg. after 8 days ($P < 0·01$). The value for neutral fat calculated from neutral-fat fatty acid was 10·14 mg./100 mg. for the control nerves, falling to 5·24 mg./100 mg. after 4 days ($P < 0·01$) and 6·6 mg./100 mg. after 8 days ($P < 0·01$). After 8 days the concentration of neutral fat determined by either method did not differ significantly from that of the control nerves. There is an obvious discrepancy between the concentration of neutral fat estimated as glycerol and that calculated from neutral-fat fatty acid. Since the figure for neutral-fat fatty acid was derived by difference and includes errors, not only in the total fatty acid procedure, but also those in the estimation of phospholipin, cholesterol and cerebroside, it can be considered as little more than an approximation. It is reassuring to note that, despite the differences in the values for neutral fat obtained by the two methods, the general trend of the figures was the same for each. Both methods demonstrated the initial fall in the concentration of neutral fat after 4 and 8 days, followed by a return to normal values.

The concentration of total lipid was 18·36 mg./100 mg. for the control nerve and decreased steadily throughout the course of the degeneration, reaching 7·95 mg./100 mg., or 43 % of the total, after 96 days.

In Table 4 the figures for each lipid are expressed as a percentage of the figure for the control nerve of the opposite side, except for ester cholesterol where the figure is expressed as a percentage of the highest value, that of the 16th day. By presenting the data from Tables 1–3 in this manner it is possible to appreciate the rate at which each lipid disappears.

Table 4 also shows the wet weight of the degenerating nerve expressed as a percentage of the weight of the control nerve. The wet weight was greatest after 4 days ($P < 0·01$), but the figures for the wet

Table 4. *Lipids of cat nerve during Wallerian degeneration*

(Concentrations expressed as a percentage of the control nerve concentration, except for ester cholesterol)

	Control	Degenerated					
		4 days	8 days	16 days	32 days	64 days	96 days
Cerebroside	100	103	96	55	25	20	0
Total cholesterol	100	100	96	83	60	32	24
Free cholesterol	100	99	92	52	36	17	9
Ester cholesterol (expressed as a percentage of 16-day concentration)	2	4	17	100	79	48	47
Total phospholipin	100	101	99	49	22	12	10
Monoaminophospholipin	100	110	104	43	18	13	11
Lecithin	100	110	112	70	49	33	25
Sphingomyelin	100	86	94	58	28	10	7
Kephalin	100	112	101	38	9	8	8
Essential lipid	100	102	98	60	33	19	12
Myelin lipid	100	98	94	55	30	16	6
Total fatty acid	100	71	77	72	83	73	71
Neutral fat (calculated from glycerol)	100	64	53	90	108	139	96
Neutral fat (calculated from neutral-fat fatty acid)	100	52	65	79	109	97	100
Total lipid	100	87	79	70	62	65	43
Wet weight	100	134	122	126	120	105	90

weight after 8 ($P < 0.01$), 16 ($P < 0.02$) and 32 days ($P < 0.05$) were all significantly greater than those of the control nerves. After 32 days the difference between the wet weight of the degenerating and the control nerves was not statistically significant.

DISCUSSION

Previous observations. The increase in the wet weight of degenerating nerve was reported by Mott & Halliburton (1901*a*, *b*) and confirmed by May (1930) and Abercrombie & Johnson (1946*b*).

Our findings on the changes in the concentration of lipids agree well with those of previous workers. Noll (1899) reported a decrease in the concentration of 'protagon' in degenerating horse and dog nerves. It is unfortunate that this excellent work was done under the shadow of the 'protagon' controversy. There can be no doubt that 'protagon', once believed to be the mother of all brain substances, contributed greatly to our present ignorance of the chemistry of 'myelin'. Excellent accounts of this interesting controversy are those of Posner & Gies (1905) and MacLean (1918).

Noll (1899) estimated 'protagon' in nerves by the reducing substances liberated during acid hydrolysis. For the most part, he was measuring galactose liberated from the cerebroside. He reported that the total solid of an ethanol extract of a dog nerve, which had degenerated for 15 days, decreased less than the phosphorus of the extract, i.e. that the phospholipin disappeared from the nerve more rapidly than the total lipid. He also reported that 'protagon', probably cerebroside, of another nerve, which had degenerated for 16 days, decreased to 54 % of the value for the control nerve. This result can be compared with our mean cerebroside concentration of 55 % for 16-day cat nerves.

Mott & Halliburton (1901*a*, *b*) reported that the concentration of total phosphorus in a degenerating cat nerve fell to zero in 29 days. The method for phosphorus estimation must have been extremely insensitive, for our experiments indicate that considerable phospholipin remains in the nerve after 32 days. May (1930) also found a decrease in total phosphorus in degenerating rabbit nerves, greatest between the 7th and 49th day. There was still 35 % of the total phosphorus present even after 100 days. He also reported a rapid decrease of lipid P after 7 days and a considerable increase in water-soluble P throughout the whole period of the degeneration.

The composition of 'myelin'. While studying the chemical nature of the lipids of 'myelin', Johnson *et al.* (1948*b*) found that the distribution of lipids in peripheral nerve resembled that of the lipids of the white matter of the brain rather than that of the grey matter. Brain white matter and peripheral nerve, both of which are rich in myelinated fibres, differed from brain grey matter in that they contained more cerebroside, free cholesterol and sphingomyelin (Johnson *et al.* 1948*a*). Subsequently, Johnson *et al.* (1949*a*) reported that it was these same three lipids, cerebroside, free cholesterol and sphingomyelin, that distinguished the white matter of the adult brain from that of the brain of the newborn infant, where myelination is incomplete. It was suggested that these three lipids, i.e. free cholesterol and the two sphingolipids, cerebroside (or glycosphingoside) and sphingomyelin (or phosphosphingoside), rather than the phosphoglycerides, lecithin and kephalin, were the principal lipid components of the myelin sheath.

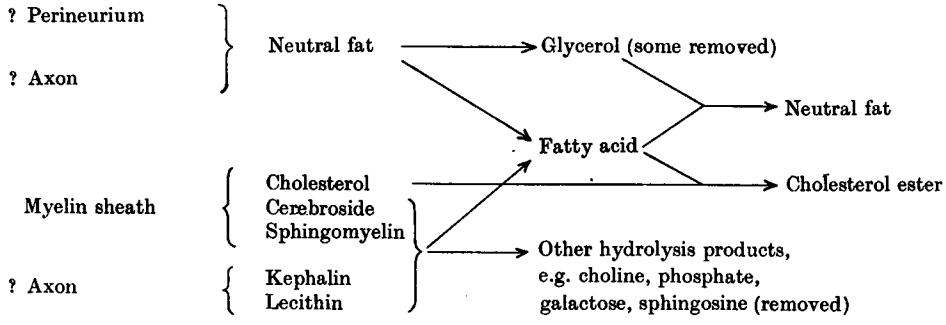
These lipids were called the myelin lipids. Carter, Haines, Ledyard & Norris (1947) found a high concentration of the sphingolipids, cerebroside and sphingomyelin, in ox spinal cord.

Each of the myelin lipids decreased in the degenerating nerve at approximately the same rate and to the same extent (Table 4). The change in the concentration of none of the other lipids resembled that of the myelin lipids; total lipid decreased much less rapidly; neutral fat decreased early, and then returned to normal; total cholesterol decreased more slowly; cholesterol ester, not present in normal nerve, appeared during the course of the degeneration; total phospholipin decreased at first more rapidly and then more slowly than the myelin lipid; lecithin decreased much more slowly and kephalin more rapidly (Table 7). The finding that cerebroside, free cholesterol and sphingomyelin decreased at the same rate and to the same extent is additional evidence supporting our previous suggestion that these substances, rather than lecithin and kephalin, are the principal lipid constituents of the myelin sheath.

Frequently the essential lipids are regarded as the important structural lipids of the body. Because a large proportion of the essential lipid of nerve is myelin lipid, they both tend to disappear from a degenerating nerve at a similar rate (Table 4). Although the essential lipids are important elements of the central nervous system as a whole, the myelin lipids would appear to play a more important role in the structure of the myelin sheath.

1939; Schmitt, Bear & Palmer, 1941). These studies indicate that the myelin sheath consists of coaxial concentric sheets of oriented lipid molecules alternating with thin, possibly unimolecular, layers of protein. The sheath shows a characteristic birefringence with the optical axes directed radially and a definite X-ray diffraction pattern. Such valuable studies tell us little, however, of the chemical nature of the lipid in this lipid-protein complex. It is suggested that this complex is rich in free cholesterol and the sphingosine-containing lipids, cerebroside and sphingomyelin.

Lipids of myelin during Wallerian degeneration. We can now give an outline of some of the changes that occur in the lipids when a peripheral nerve undergoes Wallerian degeneration. During the early stages the water content of the nerve increases, the absolute amount of the neutral fat decreases and there is little change in the myelin lipids. From 8 to 32 days there is a steady decrease in the concentration of the myelin lipids, i.e. cerebroside, free cholesterol and sphingomyelin. These substances presumably are slowly hydrolysed and the products of hydrolysis, e.g. glycerol, fatty acid, choline, galactose, sphingosine and phosphate, are removed. There is, in addition, a rapid hydrolysis of kephalin and a slower hydrolysis of lecithin. Some of the fatty acids, liberated during the hydrolysis, may combine with free cholesterol to form cholesterol ester and others may be converted into neutral fat. These changes can be represented schematically as follows:



Abercrombie & Johnson (1946*b*) found that most of the non-extractable nitrogen of rabbit nerve disappeared at a time similar to that during which we have observed the disappearance of the myelin lipid in cat nerve. This non-extractable nitrogen probably represents the so-called 'neurokeratin' (Block, 1937) and may be an important structural element of the myelin sheath.

Hitherto most of our knowledge of the constitution of the myelin sheath has come from polarized light and X-ray diffraction studies (Schmitt & Bear,

It should be stressed that the analyses were performed on the whole nerve and therefore represent not only the lipids of myelin, but also those of the axon, Schwann cells, macrophages, perineural and endoneural connective tissue and, perhaps, some epineural connective tissue also. The neutral fat is probably chiefly in the connective tissue, but some may be in the axon. The high proportion of kephalin and lecithin in grey matter of brain (Johnson *et al.* 1948*a*, 1949*a*) would suggest that these lipids are chiefly constituents of the axon.

A scheme, admittedly hypothetical, representing the demyelination process was given by Page (1937). The chief experimental evidence, quoted in support of the scheme, was the autolysis experiments of Jungmann & Kimmelstiel (1929) and Backlin (1930). The former workers found that cerebroside decreased and inorganic phosphorus increased when rabbit whole brain stood in either oxygen or nitrogen. Backlin (1930) described a fall in the concentration of free cholesterol and cerebroside, and a possible increase in the concentration of phospholipin when a rabbit brain stood for 24 hr. However, he presented the results of one experiment only. Johnson, McNabb & Rossiter (1949*c*) found no change in the concentration of cerebroside or total cholesterol in slices of cat brain which were incubated in a buffer for periods of time up to 14 days.

The text-book description of the chemistry of demyelination, that 'myelin' is composed of phospholipin which is converted into triglyceride, is probably derived from the views of Mott & Halliburton (1901*a*, *b*). That phospholipin is converted into triglyceride may be true in part, but phospholipin hydrolysis and triglyceride formation are only two of a number of changes that occur. In the past, undue emphasis has been placed on these two processes (Mott & Barratt, 1899; Setterfield & Sutton, 1935).

The period of greatest destruction of 'myelin' (8–32 days) is characterized by a decrease in the concentration of the myelin lipids, an increase in the concentration of ester cholesterol, and a return to normal of the concentration of neutral fat. During this time macrophages appear along the length of the degenerating nerve and there is a great proliferation of the Schwann cells (Holmes & Young, 1942; Young, 1942; Abercrombie & Johnson, 1946*a*). It is possible that either the macrophages or the Schwann cells contribute enzymes to aid the degradation of the lipids. The histological evidence suggests that quite large pieces of organized 'myelin' are engulfed by the macrophages and subsequently degraded. The 'myelin' is most likely destroyed while the macrophages are in the degenerating nerve, although the possibility that the macrophages remove themselves and destroy the 'myelin' elsewhere cannot be excluded. The remarkable similarity of the disappearance curves for each of the individual myelin lipids would favour the theory of particulate ingestion followed by rapid hydrolysis or removal. Johnson *et al.* (1949*c*) showed that, when brain tissue was autolysed *in vitro*, phospholipin, chiefly kephalin and sphingomyelin, was destroyed, but that there was no destruction of cerebroside or cholesterol. This would indicate that the *in vitro* degradation of the myelin lipids is different from their *in vivo* degradation. Possibly enzymes, other than those present in the non-degenerating nervous system, e.g. those of the macrophages or the Schwann cells, are necessary for complete breakdown of the myelin lipids.

Our findings are also of interest because the quantitative changes in the concentration of lipids in a degenerating nerve can be correlated with the results of previous physiological and biological investigations of Wallerian degeneration. A discussion of our results in the light of such studies will have to be postponed for a subsequent paper.

SUMMARY

1. The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin, total fatty acid and neutral fat was determined in degenerating cat sciatic nerves at intervals of time from 4 to 96 days after nerve section. From these figures the concentration of ester cholesterol, sphingomyelin, kephalin, essential lipid, myelin lipid, and total lipid was calculated.

2. The wet weight of the degenerating nerve increased rapidly, reaching a maximum in 4 days and returning to normal after 64 days.

3. The total lipid content of the nerve decreased steadily throughout the course of the degeneration.

4. Neutral fat decreased rapidly, reaching a minimum between 4 and 8 days, and returned to normal by 32 days.

5. The myelin lipids (cerebroside, free cholesterol and sphingomyelin) changed little during the first 8 days and then decreased rapidly, and to the same extent, between 8 and 32 days.

6. Cholesterol changed little during the first 8 days. Between 8 and 32 days free cholesterol decreased rapidly and ester cholesterol, absent in control nerves, increased, reaching a maximum by 16 days.

7. Total phospholipin, which changed little during the first 8 days, decreased rapidly between 8 and 32 days. Sphingomyelin decreased at a similar rate, whereas kephalin decreased more rapidly and lecithin more slowly.

8. The results are discussed with reference to the chemical nature of the lipids of 'myelin' and to the changes that occur in the lipids when a nerve undergoes Wallerian degeneration.

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Determination of *p*-Aminosalicylic Acid as *m*-Aminophenol

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Several methods have been described for the estimation of *p*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid) in blood (Lehmann, 1946; Ragaz, 1948; Tennent & Leland, 1948, 1949; Klyne & Newhouse, 1948). None of the procedures, developed in connexion with therapeutic trials of this drug in tuberculosis, were micromethods; our aim was to develop a method suitable for use with capillary blood. Bratton & Marshall's (1939) estimation of sulphonamides in blood seemed an obvious choice. Such a method was used (but not described) by McClosky, Smith & Frias (1948). These workers recorded erratic results with *p*-aminosalicylic acid, but obtained quantitative linear curves by allowing solutions of the drug to stand for several days at room temperature, or by heating them at 100° for 30–60 min.

Aqueous solutions of *p*-aminosalicylic acid are known to be decarboxylated on standing or heating (Venkataraman, Venkataraman & Lewis, 1948; Rosdahl, 1948; Oberweger, Seymour & Simmonite, 1948). The findings of McClosky *et al.* (1948) could be explained as the decarboxylation of *p*-aminosalicylate followed by diazotization of *m*-aminophenol. We thought that such a technique could be used for the micro-estimation of *p*-aminosalicylic acid and set about establishing conditions for complete decarboxylation.