The Action of Snake Venom, Phospholipase A and Trypsin on Purified Myelin *in vitro*

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1. Purified myelin was incubated with snake venom or phospholipase A in the presence of or absence of trypsin at 37°C, pH 7.4, for different times. 2. Analysis of the myelin pellet obtained after centrifugation of the myelin sample incubated with snake venom or phospholipase A alone showed conversion of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine into their corresponding lyso compounds. No significant loss of myelin protein was observed in these samples. 3. A marked digestion of basic protein and proteolipid protein was observed from the myelin pellet when trypsin was present in the incubation mixture. 4. The digestion of basic protein and particularly of proteolipid from myelin suggests that phospholipases may make protein more exposed to proteolytic enzyme for its digestion. 5. The relevance of the co-operative effect of phospholipases and proteinases as a model system of the mechanism of myelin breakdown in degenerative brain diseases is discussed.

Radioisotopic studies of myelin constituents indicate that at least part of the structure is metabolically rather stable (Davison, 1961; Smith, 1972; Sabri *et al.*, 1974; Agrawal *et al.*, 1976). However, in multiple sclerosis and other demyelinating conditions there is primary dissolution of the myelin lamellae, with early loss of basic protein. As this protein is susceptible to proteolysis, proteinases have been implicated in the demyelinating process (Einstein *et al.*, 1972; Adams *et al.*, 1971). Previous studies on isolated myelin showed that the basic protein was partially lost on treatment with trypsin, but unexpectedly the myelin-sheath ultrastructure appears to be unaltered (Raghavan *et al.*, 1973; Banik & Davison, 1974; Wood *et al.*, 1974).

Since phospholipase A incubated with isolated myelin causes changes in its lipid composition (Coles *et al.*, 1974), we have investigated the possibility that phospholipases, together with proteolytic enzymes, may cause the more complete destruction of the myelin sheath. Thus the purpose of the present work was to study the co-operative effect of phospholipases and proteinases on the dissolution of the myelin membrane in the hope that it will provide an experimental model for the degenerative process. A preliminary report of this work has appeared elsewhere (Banik & Davison, 1975).

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Experimental

Materials

Acetylated trypsin, lysophosphatidylcholine, crude snake (*Naja naja*) venom and purified phospholipase A were obtained from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). All other chemicals were AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.).

Methods

Preparation of myelin. Adult Wistar rats of either sex were used throughout these experiments. Rats were anaesthetized with chloroform before exsanguination. Brains were quickly removed, weighed and transferred into ice. The tissue was homogenized in 0.32M-sucrose and purified myelin was prepared as described by Norton (1971).

Incubation of myelin. Purified myelin was suspended in water. The suspended myelin was incubated with crude snake venom (10–150 µg/mg of myelin protein), lysophosphatidylcholine (20 µg–1.0 mg/mg of myelin protein) and phospholipase A (80 µg/mg of myelin protein) in the presence or absence of acetylated trypsin (10–25 µg/g of myelin protein) in 50mM-Tris/HCl buffer, pH 7.4 (Coles *et al.*, 1974), at 37°C with constant shaking. Myelin with or without trypsin, lysophosphatidylcholine or snake venom or phospholipase A at zero time served as controls. After the incubation the experimental and control tubes were quickly chilled in ice and centrifuged at

12000g for 10 min. A firm myelin pellet and supernatant were obtained on centrifugation and were analysed.

Determination of protein and adenosine 2':3'-cyclic monophosphate 3'-phosphodiesterase (EC 3.1.4.16) activity. Protein was determined by the method of Lowry *et al.* (1951), with albumin as standard, and adenosine 2':3'-cyclic monophosphate 3'-phosphohydrolase activity was measured by the method of Banik & Davison (1969).

Lipid extraction and separation. Lipid was extracted by the method of Folch *et al.* (1957) and was separated by t.l.c. as described previously (Banik & Davison, 1971). Lipids were separated by t.l.c. in the solvent system chloroform/methanol/aq. 12% (w/v) NH_3 (17:7:1, by vol.). In this system the lysoethanolamine phosphoglyceride was found to co-migrate with sphingomyelin, lysophosphatidylcholine and phosphatidylinositol; lysophosphatidylserine moved as a separate band. When plates were stained with iodine vapour the loss of phosphoglyceride and concomitant appearance of darkly stained bands for corresponding lyso compounds were observed (see Plate 2). Lysophosphatidylcholine was also separated by t.l.c. by the method of Coles *et al.* (1974).

Gel electrophoresis. Electrophoresis of the delipidized samples in a sodium dodecyl sulphate medium was carried out by the method of Banik *et al.* (1974). Gels were stained with Coomassie Brilliant Blue overnight and de-stained as described by Agrawal *et al.* (1972). After de-staining gels were scanned in a u.v. spectrophotometer at 595 nm fitted with a scanner.

Electron microscopy. The pelleted fractions were fixed overnight in 4.0% (w/v) glutaraldehyde in 0.1M-potassium phosphate buffer, pH 7.4, then washed three times in the same buffer and fixed in 1.0% (w/v) OsO_4 for 2 h.

Results

Effect of lysophosphatidylcholine, snake venom and phospholipase A in the presence or absence of trypsin on incubated myelin

In our experiments, when myelin preparations were incubated for 60 min in Tris/HCl buffer at 37°C, some digestion of both basic proteins occurred, suggesting the presence of an endogenous proteinase. All our experiments were therefore repeated in duplicate and data were corrected for changes in control preparations. No apparent loss of membrane protein occurred when myelin was incubated for different time-intervals separately with either lysophosphatidylcholine A. A 9% loss of protein from myelin was observed when it was incubated with snake venom alone. However, there was a marked loss of protein (17%) compared with controls

when myelin was incubated with crude snake venom in the presence of acetylated trypsin (Table 1). Digestion, particularly of basic protein, was observed in these samples in the presence of trypsin, and the appearance of faster-moving protein bands was noted. This loss of protein was greater (25%) when the concentration of snake venom and trypsin was increased or the time of incubation extended (Table 1). An extensive digestion of high-molecular-weight Wolfram protein was evident from the electrophoretic pattern of incubated samples treated with either phospholipase A or snake venom. When trypsin was incubated for 30 min with myelin previously exposed to snake venom, the loss of protein was 25%. In experiments in which both phospholipase A and trypsin were present, extensive loss of proteolipid protein and basic protein from myelin preparations resulted. The loss of basic and especially proteolipid protein appeared to be greater when myelin preincubated with snake venom or phospholipase A was further incubated with trypsin. The digestion of proteolipid protein compared with controls was 60%, and both high- and low-molecular-weight basic proteins were extensively degraded when myelin was incubated with either phospholipase A or snake venom in the presence of trypsin. The extent of digestion of high-molecular-weight basic protein was higher in the presence of phospholipase A than with snake venom (Table 2). A similar amount of low-molecular-weight basic protein was digested in the presence of either snake venom or phospholipase A.

Morphology

Electron-microscope observations of the washed myelin pellet after treatment with snake venom or phospholipase A did not reveal any structural difference compared with controls, and the myelin lamellae remained tightly packed. However, the washed myelin residues after treatment with trypsin together with phospholipase A or snake venom revealed less densely packed myelin. There was extensive splitting of myelin lamellae at the intra-period line and numerous dissociated single lamellae or free strands were also present (Plate 1). The periodicity of the myelin lamellae, trypsin- and phospholipase A-treated and control samples remained unaltered.

Effect on myelin 2':3'-cyclic phosphohydrolase activity

The total phosphohydrolase activity remained unchanged when myelin was incubated with lysophosphatidylcholine, snake venom or phospholipase A. However, a 15–20% loss of enzyme activity was observed when trypsin was incubated with these reagents (Table 1).

EXPLANATION OF PLATE I

Electron micrograph of the myelin pellet obtained after incubation of myelin with phospholipase A in the presence of acetylated trypsin

Extensive splitting and dissociation of the myelin lamellae can be seen after incubation with trypsin and phospholipase A. In normal rat myelin fractions after incubation in buffer alone, splitting of the lamellae is minimal and few single membrane vesicles are present. Sections were 70–80 nm thick. The horizontal bar represents 0.5 μ m.

EXPLANATION OF PLATE 2

T.L.C. of lipids extracted from myelin residue obtained after incubation of myelin with either snake venom or phospholipase A in the presence or absence of trypsin

The method of separation of lipids is described in the text. 1, Myelin with snake venom ($100\mu\text{g}/\text{mg}$ of myelin protein) at 0min; 2, as 1, plus trypsin ($15\mu\text{g}/\text{mg}$ of myelin protein); 3, as 2 at 60min; 4, myelin with trypsin ($15\mu\text{g}/\text{mg}$ of myelin protein) at 60min; 5, myelin with phospholipase A ($80\mu\text{g}/\text{mg}$ of myelin protein) at 0min; 6, same as 5 at 60min; 7, lipids from whole brain. Abbreviations: Chol, cholesterol; Cereb, cerebrosides; PE, ethanolamine phospholipid; PC, phosphatidylcholine; Sph, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol. In this solvent system lyso-PE, lyso-PC and lyso-PS in samples 1, 2, 3, 5 and 6 moved with sphingomyelin, PS and PI respectively. The mobility of PE, PC, sphingomyelin, PS and PI can be seen in samples 4 and 7.

Table 1. *Loss of protein and 2':3'-cyclic AMP phosphohydrolase activity on incubation of purified myelin with snake venom, phospholipase A and lysophosphatidylcholine in the presence or absence of acetylated trypsin*

Purified myelin alone incubated in buffer for 60min, and also myelin under various conditions at zero time, served as controls. *, Myelin preincubated with snake venom for 60min was further incubated with trypsin for 30min; †, myelin preincubated with snake venom for 60min was pelleted and the pellet was incubated with trypsin for 30min.

Conditions	Incubation time (min)	Total protein in myelin residue (mg/sample)		Loss of protein (% of control value)	2':3'-Cyclic AMP phosphohydrolase (μ mol of product/ h per sample)		Loss of enzyme activity (% of control value)
		0	60		0	60	
Incubation of purified myelin with:							
1. Lysophosphatidylcholine (20 μ g/mg of myelin protein)		2.56	2.52	1.6	3738	3532	5
Lysophosphatidylcholine (20 μ g/mg)+trypsin (10 μ g/mg of myelin protein)		2.63	2.32	12.0	4576	4068	12
2. Snake venom (100 μ g/mg of myelin protein)		2.26	2.06	9.0	4219	3872	8
Snake venom (100 μ g/mg)+trypsin (15 μ g/mg of myelin protein)		2.36	1.96	17.0	4366	3360	23
3. Phospholipase A (80 μ g/mg of myelin protein)		2.44	2.36	3.3	3986	3740	6
Phospholipase A (80 μ g/mg)+trypsin (15 μ g/mg of myelin protein)		2.38	2.00	16.0	4135	3167	14
4. Snake venom (150 μ g/mg of myelin protein)		1.10	1.00	9.0	1907	1812	5
Snake venom (150 μ g/mg)+trypsin (25 μ g/mg of myelin protein)		1.17	0.87	25.6	—	—	—
*Snake venom (150 μ g/mg)+trypsin (15 μ g/mg of myelin protein)		0.92	0.70	24.0	2138	1590	25
†Snake venom+trypsin (150 μ g/mg)+trypsin (15 μ g/mg of myelin protein)		0.92	0.71	24.0	2079	1542	26
5. Trypsin (15 μ g/mg of myelin protein)		0.92	0.81	12.0	1987	1620	18

Table 2. *Loss of myelin proteins on incubation with snake venom, phospholipase and lysophosphatidylcholine in the presence or absence of acetylated trypsin*

Results are expressed as percentage loss of different protein compared with control. The symbols * and † are as in Table 1.

Conditions	Incubation time (min)	Loss of myelin proteins (% of control value)			
		Wolfram protein	Proteolipid protein	Basic protein (large)	Basic protein (small)
Myelin incubated with:					
1. Snake venom (100 $\mu\text{g}/\text{mg}$ of myelin protein)	60	15	<5	<5	<5
Snake venom (100 $\mu\text{g}/\text{mg}$)+trypsin (15 $\mu\text{g}/\text{mg}$ of myelin protein)	60	85	60	42	76
2. Phospholipase A (80 $\mu\text{g}/\text{mg}$ of myelin protein)	60	10	<5	<5	<5
Phospholipase A (80 $\mu\text{g}/\text{mg}$)+trypsin (15 $\mu\text{g}/\text{mg}$ of myelin protein)	60	54	46	66	69
3. Snake venom (150 $\mu\text{g}/\text{mg}$ of myelin protein)	60	20	<5	7	11
Snake venom (150 $\mu\text{g}/\text{mg}$)+trypsin (15 $\mu\text{g}/\text{mg}$ of myelin protein)	*	71	66	68	74
	†	58	61	63	70
4. Lysophosphatidylcholine (20 $\mu\text{g}/\text{mg}$ of myelin protein)	60	7	<5	<5	<5
Lysophosphatidylcholine (20 $\mu\text{g}/\text{mg}$)+trypsin (10 $\mu\text{g}/\text{mg}$ of myelin protein)	60	15	8	25	28

Effect of phosphatidylcholine on myelin lipids

Lysophosphatidylcholine had a less marked effect than crude venom enzyme or pure phospholipase A on the composition of myelin lipids. On incubation with lysophosphatidylcholine no significant change in the turbidity of myelin was found compared with controls. Although the higher amount of lysophosphatidylcholine (1 mg/mg of myelin protein, incubated for 14 h) had an effect on myelin proteins, the effect was less than that obtained with crude snake venom or pure phospholipase A.

Action of crude snake venom and phospholipase A on myelin lipids

Although there was some loss of lipid found in the samples treated with trypsin alone, no formation of lyso compounds was detected.

When myelin preparations were incubated with either crude snake venom or phospholipase A in the presence or absence of trypsin, the myelin phospholipids, ethanolamine-containing phospholipids, phosphatidylcholine and phosphatidylserine were found to have been converted into the corresponding lyso compounds (Plate 2). The relative rates of hydrolysis of phosphoglycerides to lysophosphoglycerides in the membrane were phosphatidylserine > phosphatidylcholine > ethanolamine phospholipid. The treatment of myelin (1 mg of myelin protein) with snake venom (100 μ g) showed that 74% of phosphatidylcholine, 58% of ethanolamine phosphoglyceride and 83% of phosphatidylserine were cleaved, and with phospholipase A (80 μ g), 57% of phosphatidylcholine, 40% of ethanolamine phosphoglyceride and 63% of phosphatidylserine were hydrolysed compared with the control. Most of the lysophosphoglycerides were present in the pellet obtained after centrifugation of the incubated myelin sample, and only a negligible amount of lysophosphatidylcholine could be demonstrated in the supernatant fraction on t.l.c. Thus phospholipase A present in the crude snake venom was active for the conversion of myelin phosphoglycerides into their lyso derivatives, whereas galactolipid and cholesterol contents remained unchanged. The change observed in cholesterol and cerebroside concentration after incubation with either snake venom (100 μ g) or phospholipase A (80 μ g) was less than 5% compared with the control.

No complete hydrolysis of myelin phosphoglycerides was obtained even when the amount of crude snake venom was increased to 100–150 μ g/mg of myelin protein. Under these experimental conditions the extent of hydrolysis was greater than that found with lesser amount of crude venom (20 μ g/mg of myelin protein). The rate of hydrolysis of phosphoglycerides was obtained by incubating myelin at different times either with snake venom

(20 μ g) or phospholipase A. Phosphatidylserine was hydrolysed more rapidly than phosphatidylcholine and ethanolamine phospholipid, and phosphatidylcholine was hydrolysed faster than ethanolamine phosphoglyceride.

Discussion

Since it has been proposed that proteolytic enzymes are involved in the breakdown of the myelin sheath in demyelinating diseases (Einstein *et al.*, 1969; Hallpike *et al.*, 1970; Ramsey *et al.*, 1974; Smith & Rauch, 1974), we have previously taken the effect of a proteolytic enzyme, trypsin, on myelin *in vitro* as a possible model system (Banik & Davison, 1974; Wood *et al.*, 1974). Although our studies with trypsin showed the loss of lipids, including neutral lipid, and basic encephalitogenic protein from myelin, there was unexpectedly no alteration in the ultrastructure of the myelin sheath. Wood *et al.* (1974) had noted the same in their experiments. We therefore extended this study by adding phospholipase A or crude snake venom to our incubation medium in the presence of trypsin, to evaluate the combined effect of these enzymes on the dissolution of myelin. When isolated myelin is incubated with either lysophosphatidylcholine or phospholipase A, there is no apparent loss of protein (small corrections are made for endogenous myelin proteinase activity). In the presence of trypsin there is a 15–30% loss of protein from the membrane. After incubation of myelin with phospholipase A or snake venom in the presence of trypsin, this loss of myelin protein is shown to be due to digestion not only of basic protein but also of proteolipid protein.

The lipid profile of the pelleted myelin fractions showed a loss of all classes of lipids and also showed the conversion of myelin phosphoglycerides into their corresponding lyso compounds. Lysophospholipids were found to have remained with the pelleted myelin membrane, and only small amounts were detectable in the supernatant. These results are in agreement with Coles *et al.* (1974), where they incubated myelin preparations with phospholipase A.

There is evidence from the findings of Poduslo & Braun (1973) that basic protein is localized on the cytoplasmic side (dense period line) of the myelin and is therefore available in myelin preparations to tryptic digestion, whereas proteolipid protein may be protected by its hydrophobic lipid environment (Folch, 1971). Once these lipids are removed the proteolipid protein becomes exposed to proteolytic attack, leading, it is postulated, to the disintegration of the membrane.

The disintegration of the myelin sheath was observed in the electron micrograph of the incubated myelin sample, where splitting of the myelin lamellae was evident (Plate 1). After a split

of the intraperiod line or dense line, the peeled-off myelin lamellae was found to have formed vesicular structures. This type of dissolution of the myelin sheath has been demonstrated in experimental allergic encephalomyelitis (Lampert & Carpenter, 1965; Lampert & Kies, 1967). The vesicular myelin debris as well as the part of the intact sheath are probably later removed by activated macrophages in the diseased condition.

Elevated activities of phospholipase have since been demonstrated in tissues from patients with experimental allergic encephalomyelitis and also in tissues from patients with multiple sclerosis (Woelk & Kanig, 1974; Woelk & Peiler-Ichikawa, 1974). Increased proteinase has also been found in experimental allergic encephalomyelitis and demyelinating tissues, both histochemically and biochemically, by various investigators (Einstein *et al.*, 1969; Hallpike *et al.*, 1970; Cuzner & Davison, 1973; Ramsey *et al.*, 1974). In view of these findings, phospholipase and proteinases may be jointly involved in the degradation of the myelin sheath in demyelinating diseases. These hydrolases present in activated macrophages (David, 1975) may well be responsible for the primary attack on the myelin sheath in the demyelinating process.

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