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Short Communications

Criteria for Identifying a Myelin-like Fraction from Developing Brain

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Multiple criteria must be used to relate a membrane fraction ('myelin-like') to myelin. These reported in this communication are electron microscopy, marker enzymes, sodium dodecyl sulphate–polyacrylamide-gel electrophoretic patterns and lipid composition.

Myelin is a complex lipid and lipid–protein aggregate which ensheaths axons. It undergoes profound morphological (Peters & Vaughn, 1970) and biochemical changes (Horrocks, 1968; Cuzner & Davison, 1968; Eng & Noble, 1968; Dalal & Einstein, 1969; Norton, 1971; Agrawal & Davison, 1973) during development. Davison and co-workers (Banik & Davison, 1969; Agrawal *et al.*, 1970) have isolated a membrane fraction termed 'myelin-like' from myelin isolated from 15-day-old rat brain. The chemical composition and morphological characteristics of myelin-like fraction were quite different from purified myelin (Agrawal *et al.*, 1970). The present communication in general confirms the observation of Agrawal *et al.* (1970) and extends these observations to demonstrate clearly the presence of basic protein in myelin-like material, a protein highly characteristic of myelin. Although this observation may support the earlier suggestion of Davison and co-workers that this membrane may be a precursor of myelin, it is essential to remember that several different criteria must be used to relate myelin with other membrane fractions.

Eight rat brains (15 days old; Sprague–Dawley variety, from Zivic–Miller Laboratories, Pittsburgh, Pa., U.S.A.) were used for each fractionation by the method of Agrawal *et al.* (1970). The myelin-like fraction and other subcellular fractions were prepared essentially by the technique of Agrawal *et al.* (1970) with four modifications. (1) Brains were homogenized by hand in an all-glass Dounce homogenizer as described by Norton (1971). (2) Ca^{2+} was not added to the sucrose solution. (3) The crude myelin was osmotically 'shocked' with 9 vol. of water for 20min at 0–4°C. (4) A 'large microsomal' fraction was removed from the microsomal fraction by centrifuging the suspended microsomal fraction at 17000*g* and collecting the resultant pellet. All fractions were diluted with water and centrifuged for 30min at 100000*g*. The pellets were resuspended in water and stored at −75°C until needed for enzymic or other chemical analysis. The results of enzymic analysis are the mean of three separate fractionations. The sodium dodecyl sulphate–polyacrylamide-gel electrophoretic protein pattern is typical of those obtained from three separate subcellular fractions. Protein was determined by the method of Lowry *et al.* (1951) and as modified by Hess and Lewin (1965).

The purified myelin contained large saccular pieces of multilamellar myelin together with some smaller vesicles (Plate 1*a*). The myelin-like material consisted mostly of vesicles, whose walls were unit membranes. Some were large enough to be termed sacs rather than vesicles (Plate 1*b*). A careful electron-microscopic scanning of myelin-like material from a number of preparations indicated the infrequent presence of very small pieces of myelin fragments. The microsomal fraction also contained vesicles with unit-membrane walls (Plate 1*c*). Many of these vesicles contain various amounts of an apparently fibrillar material. Granular microsomes were virtually absent, although a considerable number of free ribosomes were present (Plate 1*c*).

The lipid compositions of the myelin and myelin-like fractions were determined on extracts combined after consecutive extractions of the membrane with ether–ethanol (3:2, v/v) and chloroform–methanol (2:1, v/v). The lipids were partitioned by the method of Folch *et al.* (1957). The purified myelin had molar proportions of cholesterol:phospholipid:cerebrosides of 1:1.35:0.3, compared with the respective values for the myelin-like fraction of 1:1.74:0 (or very low). These values are comparable with those

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reported by Agrawal *et al.* (1970). The primary differences in lipids between the two fractions, i.e. myelin and myelin-like, resides in the higher total phospholipids and the very low amount or absence of cerebrosides for the myelin-like fraction. Since myelin contains a greater percentage of total lipids than the myelin-like fraction, this accounts for the differences in densities of these two membrane fractions.

Three marker enzymes were measured to ascertain the 'purity' of myelin-like and purified myelin fractions. These were 2′:3′-cyclic nucleotide 3′-phosphohydrolase [by the method of Kurihara & Tsukada (1967), as modified by Banik & Davison (1969)], acetylcholinesterase (EC 3.1.1.7; by the acetylthiocholine procedure of Ellman *et al.*, 1961) and β-hydroxybutyrate dehydrogenase (EC 1.1.1.30; by the method of Williamson *et al.*, 1971). The activity of 2′:3′-cyclic nucleotide 3′-phosphohydrolase in myelin-like and purified myelin fractions respectively was 764±76 and 1214±108 (S.E.M.) μmol of 2′-AMP produced/h per mg of protein. The activity of this enzyme in microsomal fractions, mitochondria and nerve-ending particles was between 5 and 25% of that in purified myelin. In contrast the activity of acetylcholinesterase, which is localized chiefly in microsomal fractions and nerve endings, was high in myelin-like preparations (8.25± 0.13 μmol of acetylthiocholine hydrolysed/h per mg of protein), and was lower in purified myelin (2.53± 0.38). This could indicate extensive microsomal contamination of the myelin-like fractions; on the other hand, acetylcholinesterase may be an integral part of the myelin-like fraction itself. It must be emphasized that the specific activity of the phosphohydrolase in microsomal fractions was only 4 and 6% of that of purified myelin and myelin-like material respectively. The activity of β-hydroxybutyrate dehydrogenase, an enzyme characteristic of mitochondria, was essentially undetectable in myelin-like material. In purified myelin the activity of this enzyme (sp. activity 9.2±2.9 μmol/min per g of protein) was 10% of that of mitochondrial fraction.

Plates 2(*a*) and 2(*b*) present the gel-electrophoretic pattern of 'large microsomes', microsomal fractions, mitochondria, nerve-ending particles, myelin-like material and purified myelin. The protein profile of purified myelin was distinguishable from that of other subcellular fractions, including myelin-like material. There was a preponderance of high-molecular-weight proteins in myelin-like material, a finding confirming earlier observations (Agrawal *et al.*, 1970; Morell *et al.*, 1972; Benjamins *et al.*, 1973; Adams & Osborne, 1973; Poduslo & Norton, 1973). Of special interest is the fact that myelin-like material gives two bands whose mobilities are similar to those of myelin basic protein, an observation consistent with that of Benjamins *et al.* (1973) and Adams & Osborne (1973). In addition, when the myelin-like fraction was mixed with purified myelin, the two protein bands of myelin-like fraction migrated with those of the myelin basic protein. These two protein bands in the myelin-like fraction were labelled by [2,3-^{3}H]tryptophan, eliminating the possibility that they are histones. Using younger animals as reported in the present paper, we could not identify with certainty bands in myelin-like fraction corresponding to proteolipid protein and fraction DM-20 (Agrawal *et al.*, 1972) (Plates 2*a* and 2*b*).

In this communication we have used several criteria to identify myelin-related subfractions, i.e. electron microscopy, enzyme profiles, lipid analysis and sodium dodecyl sulphate–polyacrylamide-gel electrophoretic protein profiles. If only one criterion has been used, for example polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, the protein profiles of a number of membrane fractions appear similar (compare patterns for myelin-like, nerve-ending, microsomal and mitochondrial proteins). Therefore it would not be possible to establish a clear-cut relationship between myelin and any other membrane fraction (Plates 2*a* and 2*b*). However, when a number of criteria are used, such as those presented in this paper, it is possible to conclude that myelin-like material is related to myelin and the other subfractions studied are not.

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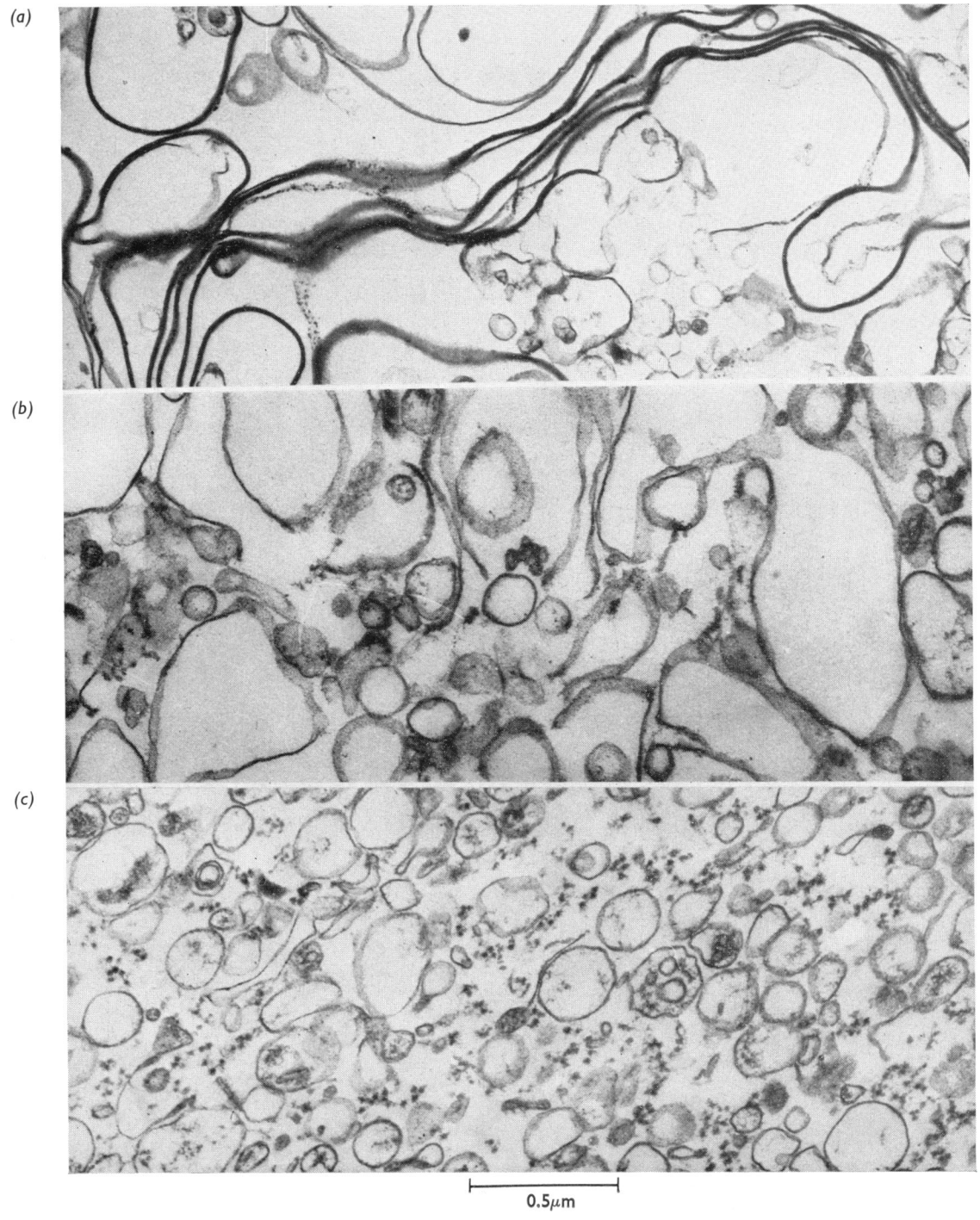
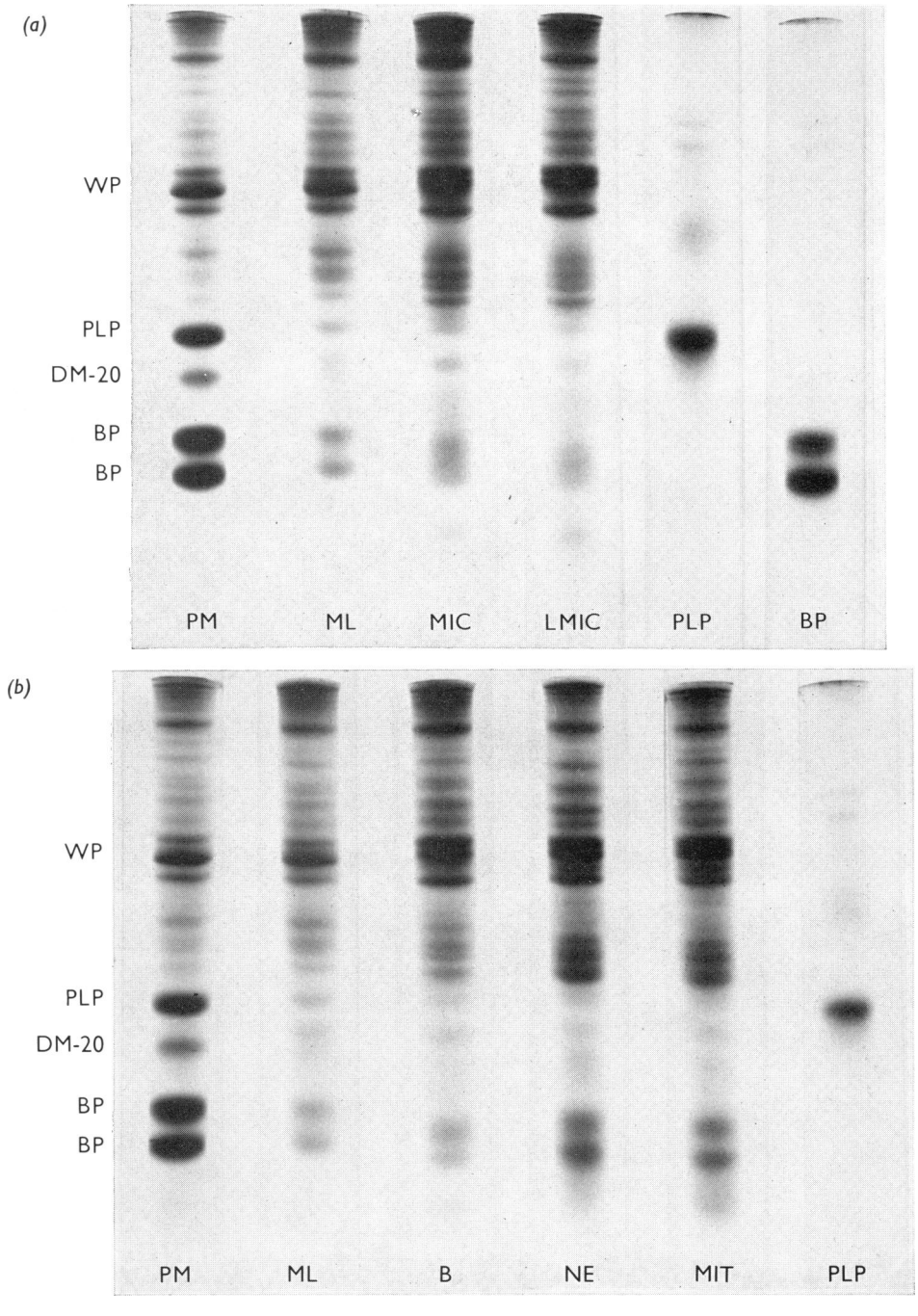


EXPLANATION OF PLATE 1

Electron micrographs of fractions from 15*-day-old rat brain prepared by the technique of Agrawal et al.* (1970) Electron microscopy was performed as described previously (Agrawal *et al.*, 1972). (*a*) Purified myelin; (*b*) myelin-like material; (*c*) microsomal fraction. Magnification ×45600.


(a)
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(b)
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EXPLANATION OF PLATE 2

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of proteins from various fractions of rat brain

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out, as described by Agrawal *et al.* (1972), in glass tubes of internal diameter 6mm and length 150mm. The membrane fractions were partially delipidated by repeated suspension in ether–ethanol (3:2, v/v) and centrifugation, with a final wash in anhydrous diethyl ether. The residue was dissolved in sample buffer [5mM-sodium phosphate buffer, 8% (w/v) sucrose and 1% (w/v) sodium dodecyl sulphate, pH7.2]. Suitable samples were applied to each gel to provide 40μg of protein from each membrane fraction. The current was 0.5mA/gel for 1h and was then increased to 1.0mA/gel, which was maintained for 15–18h. It was then increased to 4mA/gel for 2h. The gels were stained for 15–24h in 0.25% Coomassie Brilliant Blue dissolved in 45% (v/v) methanol–10% (v/v) acetic acid. Destaining was carried out by diffusion as described by Agrawal *et al.* (1972). Standards used for BP (basic proteins) and PLP (proteolipid protein) were prepared by preparative sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Other abbreviations used are: WP, 'Wolfgram protein' (Wolfgram, 1966); DM-20 (see Agrawal *et al.*, 1972). (*a*) PM, purified myelin; ML, myelin-like fraction; MIC, microsomal fraction; LMIC, light or low-density microsomal fraction; PLP and BP standards. (*b*) PM; ML; B, B fraction of De Robertis *et al.* (1962); NE, nerve-ending-enriched fraction; MIT, mitochondrial fraction; and PLP standards.