1. Molecular biology of early response genes and transcriptional regulation of CNS development and function.

2. Molecular determinants of neural regeneration.

3. Cytokines: their relation to neurotrophic and neurite growth.

4. Molecular signalling for cell growth.

5. Role of protein tyrosine phosphorylation in neural tissues.

6. Calcium dynamics and neural function.

7. Molecular biology of myelin: the glial–neural relationship.

8. Excitability – its regulation by eicosanoids and other lipid mediators.

9. Monitoring cellular biochemistry in intact neuronal systems.

10. Ageing of the brain.

Unfortunately, the research effort in any of these areas in India is subcritical. There is no need to emphasize the importance of brain research. I wish to suggest that a 'National Committee for Brain Research' be constituted to identify and encourage research programmes in this vital field.

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X-Ray studies on the bilayer structure of trypsin-treated rat brain myelin

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Trypsin-treated rat brain myelin was subjected to biochemical and X-ray studies. Untreated myelin gave rise to a pattern of three rings with a fundamental repeat period of 155 Å consisting of two bilayers per repeat period, whereas myelin treated with trypsin showed a fundamental repeat period of 75 Å with one bilayer per repeat period. The integrated raw intensity of the $h = 4$ reflection with respect to the $h = 2$ reflection is 0.38 for untreated myelin. The corresponding value reduced to 0.23, 0.18, 0.17 for myelin treated with 5, 10, 40 units of trypsin per mg of myelin, respectively, for 30 min at 30°C. The decrease in relative raw intensity of the higher-order reflection relative to the lower-order reflection is suggestive of a disordering of the phosphate groups upon trypsin treatment or an increased mosaicity of the membrane or a combination of both these effects. However, trypsin treatment does not lead to a complete breakdown of the membrane. The integrated intensity of the $h = 1$ reflection, though weak, is above the measurable threshold for untreated myelin, whereas the corresponding intensity is below the measurable threshold for trypsin-treated myelin, indicating a possible asymmetric to symmetric transition of the myelin bilayer structure about its centre after trypsin treatment.

SEVERAL studies have been made on the susceptibility of the proteins in the myelin membrane to various proteases since proteases are thought to be involved in the etiology of demyelinating diseases such as multiple sclerosis and experimental allergic encephalomyelitis causing disruption of the myelin structure. Supporting this contention, Cammer et al.[1,2] have demonstrated that neutral proteases secreted by macrophages degrade the basic proteins in the myelin membrane. Myelin basic proteins were also shown to be markedly hydrolysed by endogeneous neutral proteases of the brain[3], myelin[4,5], serum[6] and leukocytes[7]. Therefore, it would be of interest to study the effect of proteases on the bilayer structure of myelin.

X-ray diffraction studies on a variety of myelinated nerves from both the peripheral nervous system (PNS) and the central nervous system (CNS) have been reported[8]. Similar studies on PNS myelin at 10 Å

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resolution[9], at 7 Å resolution[10] and on other myelin samples[11-13] have also been carried out. X-ray diffraction studies on the effect of protease treatment on frog sciatic nerve myelin[14] indicated that the enzyme-digested membrane moves irreversibly into the separated state or into the condensed state or into a mixture of these two states. Electron microscopy[15] of trypsin-digested mice sciatic nerve myelin showed that the main dense line was split, making it difficult to distinguish between the intraperiod and interperiod lines. However, the microscope studies of Yu and Bunge[16] on peripheral nerve myelin showed that enzyme treatment does not lead to a complete breakdown of myelin, though it damages the myelin sheath. Thus, most of the detailed X-ray and microscope studies were on PNS myelin and similar data on CNS myelin are rather meagre[17] despite the fact that CNS myelin is implicated in demyelinating diseases. We have carried out bio-chemical and small-angle X-ray scattering experiments *in vitro* on the effect of trypsin on the composition and structure of rat brain myelin; the results of this study are reported here. A preliminary account of this work has been presented in reference 18.

Materials and methods

Trypsin (TPCK-treated, 10,000 BAEE units/mg protein) was purchased from Sigma Chemical Company, USA. Nitrocellulose was obtained from Schleiser and Schul., West Germany.

Preparation of myelin

Rat brain myelin was prepared according to the method of Norton and Poduslo[19] and stored as lyophilized powder under desiccation at –20°C. In some experiments freshly prepared myelin was also used.

Incubation of myelin with trypsin

Myelin was subjected to controlled tryptic hydrolysis as described earlier[20]. Briefly, lyophilized myelin (20 mg, i.e., 5 mg of myelin protein) was suspended by mild homogenization in 2 ml of 20 mM phosphate buffer, pH 7.0, and incubated at 30°C for 30 min with the requisite amount of trypsin. After incubation, an aliquot (0.5 ml) was removed, delipidated, solubilized in the sample buffer containing 2% SDS, 50 mM Tris–HCl (pH 7.4), 10% glycerol and 1% mercaptoethanol and subjected to SDS–polyacrylamide gel electrophoresis (12%) according to Laemmli[21]. To the rest of the material, cold distilled water was added and centrifuged at 10,000 rpm in a Sorvall centrifuge using a SS-34 rotor for 5 min and the wet pellet was used for X-ray analysis.
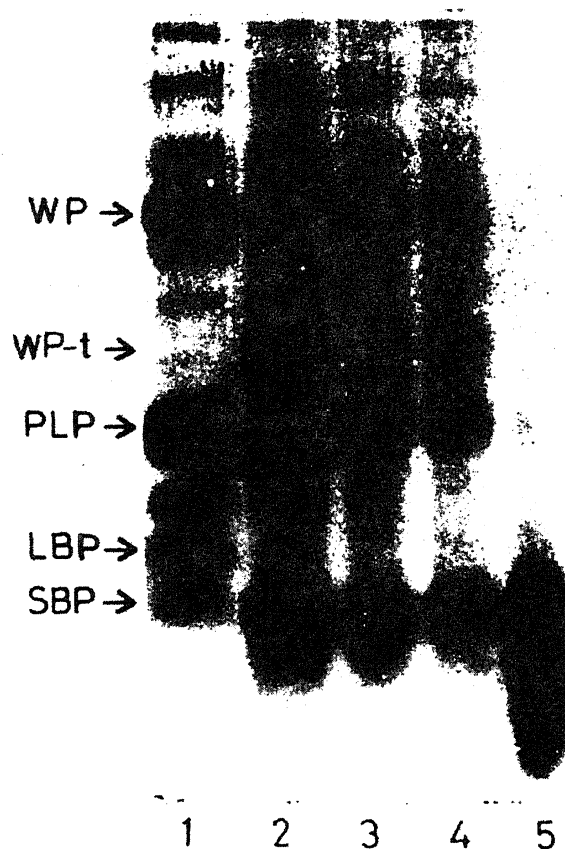

Figure 1. SDS-Polyacrylamide gel electrophoresis (12%) analysis of myelin subjected to tryptic digestion. **Lane 1,** control (without trypsin); **Lane 2–5,** trypsin-digested samples (5, 10, 20 and 40 units of trypsin/mg of myelin, respectively). WP, Wolfgram protein; WP-t, product of WP degradation by trypsin; PLP, proteolipid protein; LBP, large basic protein; SBP, small basic protein.

X-ray diffraction

The wet pellet was loaded into a 1 mm diameter thin-walled (1 mm) glass capillary tube along with a few drops of distilled water. Both the ends of the capillary were sealed with a low-melting-point sealing wax. Thus, it was ensured that all the samples are subject to diffraction under similar conditions of hydration. All the X-ray experiments were conducted at room temperature (25°C).

Small-angle X-ray diffraction patterns were recorded using a Searle small-angle camera (Marconi, Avionics, England) equipped with Frank's mirrors to focus the beam in both horizontal and vertical directions. The doubly focused beam was used for diffraction experiments, the other beams being eliminated using slits capable of moving in both the horizontal and vertical directions.

The X-ray source used was a GX-18 rotating-anode X-ray generator operated at 30 kV, 30 mA, with a 100 μm focal cup in the point focus mode. The CuKα radiation was nickel-filtered before being incident on the focusing mirrors. The specimen-to-film distance was

maintained at 98 mm using a precalibrated set of spacers. Exposure times were typically of the order of 30 h. Two circular X-ray films (A, B) were used per pack. A Carl Zeiss MD 100 microdensitometer was used for tracing the X-ray patterns. The repeat periods were measured to an accuracy of 1%. The background scattering was subtracted from the patterns and the integrated intensities were estimated by measuring the area under the curves. The films (A, B) included per pack were scaled together to yield the final set of integrated intensities. The intensity range covered in the present X-ray experiments is 200 : 1.

Results and discussion

SDS-PAGE analysis of myelin subjected to trypsin digestion

Figure 1 shows the SDS electrophoresis pattern of myelin subjected to various concentrations of trypsin. It may be noted that there is a progressive degradation of myelin proteins with increased concentration of trypsin and that there was an almost complete digestion of myelin proteins with 40 units of trypsin per mg of myelin, in 30 min (Figure 1, lane 5). It may be noted further that (lanes 2–4) the basic proteins and the Wolfgram proteins were degraded more rapidly than proteolipid proteins. A new protein band (35 k, Wp-t) has arisen from Wolfgram protein as was reported earlier[20].

To ensure that there was no further degradation of proteins during the 30 h exposure to X-rays used to obtain the X-ray diffraction pattern, the following experiment was done. Aliquots of myelin treated with various concentrations of trypsin as described above were analysed by SDS-electrophoresis immediately or after 30 h of storage at room temperature (25°C) without and with soya bean trypsin inhibitor (50 mg). These experiments indicated that no further degradation of proteins occurred after the initial incubation. Worthington et al.[14] have found that the lamellar structure of myelin at 17 Å resolution does not change due to mere incubation at 37°C for 6 h.

X-ray diffraction analysis

The X-ray photograph of the untreated myelin is shown in Figure 2. The background-subtracted densitometer tracings of the A films of untreated myelin and trypsin-treated myelin are shown in Figure 3. A comparison of the relative integrated intensities of the reflections from untreated and tryspin-treated myelins is made in Table 1.

The diffraction pattern of hydrated untreated myelin consists of three orders of diffraction with a fundamental repeat period of 155 Å and with h indices of 1, 2 and 4 (rings A, B and C with d spacings of 155 Å, 77.5 Å and 38.8 Å, respectively). The strongest reflection was from the order $h = 2$ (ring B) and the pattern is comparable to that obtained from rat optic nerve[9] and other central nervous system myelin[8]. The intensity of the reflection corresponding to the A ring was below the observable threshold in all the trypsin-treated samples. The d spacings for the B and C rings were found to be 75 Å and 37.5 Å, respectively.
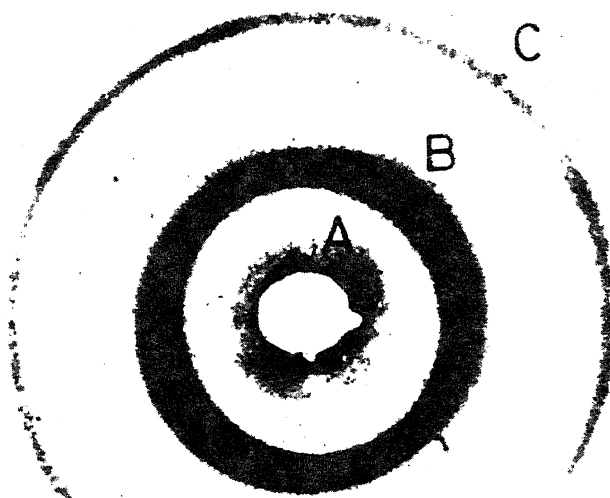

Figure 2. Small-angle X-ray diffraction photograph of hydrated untreated myelin isolated from rat brain. The most intense ring corresponds to a d spacing of 77.5 Å. The d spacings of the rings A, B and C correspond to 155 Å, 77.5 Å and 38.8 Å, respectively.
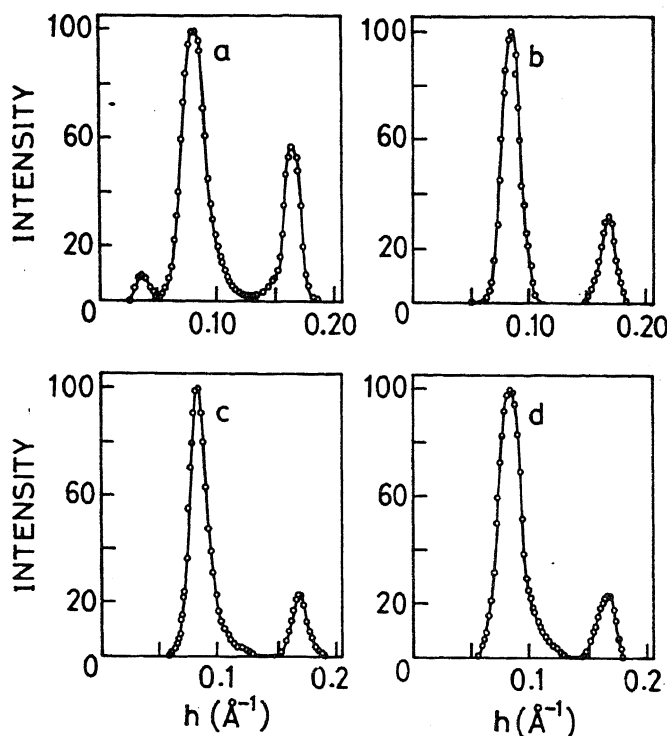
Figure 3. Background-subtracted densitometric tracings of the X-ray diffraction patterns of myelin. a, Untreated myelin; b, c and d are myelin treated with 5, 10, 40 units of trypsin/mg myelin, respectively ($h = 4\pi \sin \theta/\lambda$) ($\text{Å}^{-1}$).

Table 1. Comparison of the relative integrated raw intensities of rings A, B and C (Figure 2) of isolated rat brain myelin before and after trypsin treatment (I, untreated myelin; II, III and IV are myelin treated with 5, 10, 40 units of trypsin units/mg myelin, respectively, as described in text)

	A	B	C
I	0.04	1.0	0.38
II	*	1.0	0.23
III	*	1.0	0.18
IV	*	1.0	0.17

*Indicates that the intensity of the A ring was below the measureable threshold for all the trypsin-treated myelin samples.

grated intensity of the A ring below the measurable threshold for all trypsin-treated myelins was also noticed (Table 1) in the present experiment.

It is remarkable that the multilayer structure of isolated rat brain myelin is preserved even after trypsin treatment. The presence of the multilayer structure is confirmed by X-ray diffraction experiments, which showed the occurrence of multilayer reflections that can be indexed on a lattice. This is in accordance with the observations of Yu and Bunge[16] and Peterson[15], whose electron microscope studies showed that the damage to PNS myelin sheath caused by trypsin does not lead to a complete breakdown of the membrane. Worthington et al.[14] have also recorded multilayer reflections from enzyme-treated frog sciatic nerve. The preservation of the multilayer structure could be due to the fact that the peptide fragments of myelin protein are still retained within the membrane and these prevent a complete breakdown of the membrane structure.

It is of interest to compare the electron density profiles of untreated myelin with those of trypsin-treated myelin. Since the signs of reflections for rat brain myelin are not explicitly available, we have used the signs of reflections from rat optic nerve myelin, which is a CNS myelin of comparable fundamental repeat period (156 Å, ref. 9). The signs of the first five structure amplitudes were found to be invariant for frog sciatic nerve, rat sciatic nerve and rat optic nerve[9]. The sign assignments agree with those of McIntosh and Worthington[23], i.e., –, + and – for the orders $h = 1, 2$ and 4 of untreated myelin (A, B and C rings). The sign + and – were assigned to the first two observed reflections of trypsin-treated myelins (B and C rings).

The structure amplitudes ($|F(h)|$) were calculated from the integrated intensities $I(h)$ using the formula[9] $|F(h)| = [hI(h)]^{1/2}$. The centrosymmetric Fourier transforms were calculated and were scaled with respect to each other by the following empirical procedure. In general, $\rho_{absolute} = $ scale factor $\cdot$ ($\rho_{relative} +$ an additive constant), i.e., $\rho_{abs} + k$ ($\rho_{rel} + a$), where ρ is the electron density.

The electron density at the hydrocarbon chain may be assumed to be 0.27 $e/\text{Å}^3$ in all the samples studied and

The d spacings for the reflections from trypsin-treated myelin samples are consistently less than those from untreated myelin. From these data it appears that the 155 Å fundamental repeat period representing two bilayers is not present in trypsin-treated myelins and only the 75 Å repeat period representing one bilayer is preserved. The repeat period for two bilayers in trypsin-treated myelin is 150 Å. Apparently, the rat brain myelin moves into a condensed state upon trypsin treatment. According to Worthington et al.[14], PNS myelin membrane moves irreversibly into either the separated state or the condensed state or a mixture of these two states, the major difference between the two states being the width of the fluid layer between adjacent myelin layers.

It may be seen from Table 1 that the integrated intensity of the C ring relative to the B ring, which is 0.38 for untreated myelin, reduces to 0.23, 0.18, 0.17 for myelin treated with 5, 10, 40 units of trypsin per mg of myelin, respectively.

Small-angle X-ray diffraction studies of Wood et al.[22] on trypsin-treated myelin did not show any change in the diffraction pattern. However, our studies on the small-angle X-ray diffraction of myelin treated with various quantities of trypsin showed reproducible changes in the diffraction pattern, such as a decrease in the fundamental repeat period and in the relative intensity of the higher-order reflection (C ring) with respect to the lower-order reflection (B ring). A decrease in the inte-
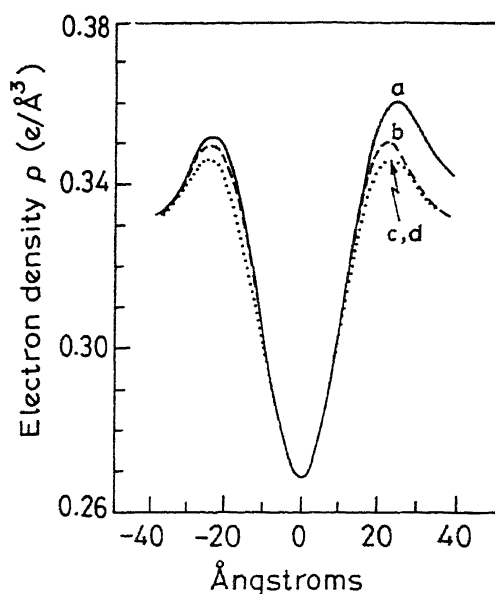
Figure 4. Electron density profiles. The origin of the unit cell is located at the centre of the hydrocarbon region: *a*, untreated myelin; *b*, *c* and *d* are myelin treated with 5, 10, 40 units of trypsin/mg myelin, respectively.

that at the interbilayer space to be 0.33 $e/Å^3$, These two conditions are sufficient to evaluate the constants K and a for any given sample. Even though this method does not provide a quantitative estimate of the electron densities, it enables at least a qualitative comparison of the electron density profiles from different samples. A similar method has been used by Murthy *et al.*[24].

The electron density profiles of the untreated myelin and those of trypsin-treated myelins are depicted in Figure 4. From a comparison of the profiles, it is clear that the electron density associated with the lipid phosphate groups has decreased for all the trypsin-treated myelins relative to the untreated myelin. Independent of the electron density profiles, the decrease in relative intensity of the higher-order reflection (C ring) relative to the lower-order reflection (B ring) for trypsin-treated myelin may be looked upon as being due to an increased disorder or thermal vibrations of the phosphate groups, which are the main scatterers of X-rays in myelin. Alternatively, trypsin treatment induces an increased mosaicity of the membrane, without, however, leading to its complete breakdown. All these effects could also occur in combination.

The absence of odd-ordered reflection ($h = 1$) (A ring) from trypsin-treated myelin suggests that CNS myelin, which is normally asymmetric, becomes symmetric upon enzyme treatment. This is in accordance with the observation of Worthington *et al.*[14] that PNS myelin (frog sciatic nerve myelin), which is normally asymmetric, becomes symmetric about its centre presumably because of a redistribution of protein components after treatment with trypsin or pronase. Such an asymmetric-to-symmetric transition of myelin membrane after enzyme

treatment was supported by electron microscopic observations also[15, 16]. However, it must be noted that the conclusions regarding the asymmetric-to-symmetric transition in rat brain myelin have been arrived at on the basis of the lowest-order odd reflection. Higher-order odd reflections were not observed even in untreated rat brain myelin.

Conclusions

X-ray diffraction studies on rat brain myelin subjected to controlled trypsin digestion *in vitro* indicated the following:

a) The fundamental repeat period of untreated rat brain myelin, which is 155 Å with two bilayers per repeat period, changes to 75 Å with one bilayer per repeat period. However, the enzyme treatment does not lead to a complete breakdown of the multilayer structure of the membrane.

b) The ratio of the relative raw integrated intensity of the higher-order reflection (C ring) with respect to the lower-order reflection (B ring) decreases upon trypsin treatment. This might be due to an increased disorder of the phosphate group or an increased mosaicity of the membrane or a combination of these effects.

c) Within the limits of accuracy available in the present experiment, rat brain myelin, which is normally asymmetric, becomes symmetric about its centre after treatment with trypsin.

It may be speculated that similar changes might occur in myelin *in vivo* when it is acted upon by enzymes in multiple sclerosis or experimental allergic encephalomyelitis. The structural and other alterations in myelin might affect its performance as an insulator and thereby impair the conduction of electrical signals in nerve fibres.

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Significance of pharmacological study of neurotransmitter receptors in brain research

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Studies of the various aspects of receptor structure and their modulation by pharmacological agents represent a major area of current thrust in neurosciences at the cellular and subcellular level as well as at the behavioural level. The pharmacological criteria for receptor characterization have been used extensively not only to classify the receptors but also to understand the physiological roles of various neurotransmitters and help in the development of new drugs. The information obtained using procedures of molecular biology is complementary and a proper characterization of a receptor needs both types of information.

The thrust area for neurotransmitter receptor research should include basic studies to obtain receptor in a pure form, techniques to solubilize them, mapping of neurotransmitter receptors in healthy and diseased brain and their interactions with drugs. Ligands need to be discovered for orphan receptors, and the suitability of using peripheral receptors in lymphocytes, platelets, etc., as well as invertebrate receptors as indicators of CNS receptors should receive top priority in research.

THE term receptor has been used by pharmacologists and physiologists for over a century, but the concept, the definition and the functions have undergone major changes during the last decade. There has been a tremendous growth in information about drug–receptor interactions and about receptor *per se*, and in 1987 the International Union of Pharmacological Sciences appointed a Committee to rationalize the nomenclature of receptors. The Committee has, in principle, accepted a broad-based operational definition of receptor. Briefly stated, a receptor must recognize a distinct chemical entity and translate this information into a form that the cell can read and alter its overt state by initiation of a biochemical process, by alteration in an ongoing biochemical process or by modulation of the action of an indigenous effector. This definition ascribes the function of both recognition and transduction to a receptor.

Historical development of the receptor concept

Waldeyer in 1891 coined the term neurone[1] and Langley in 1909 further developing the concept of drug–receptor interaction, stated that drugs could form a dissociable complex with the receptor and that receptive substances (receptors) on cells had a clearly defined physiological function[2]. Around the same time, Hill[3] analysed the action of nicotine and curare on frog skeletal muscle to provide evidence that drug action could be antagonized also by interaction at the receptor level.

Most of the early studies, even though employing pharmacological agents, were qualitative. Clark[4,5] pioneered the concept of dose–response relationship and initiated a quantitative study of drug–receptor interaction. Schild[6] further extended the quantitation to drug antagonism at receptors and used the negative log of molecular concentration of an antagonist (pAx), which would reduce the effect of multiple dose of an agonist to that of a single dose in the absence of the antagonist[7].