Detergent Activation and Solubilization of 2':3'-Cyclic Nucleotide 3'-Phosphodiesterase from Isolated Myelin and C₆ Cells

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Several detergents were investigated for their ability to increase activity of 2':3'-cyclic nucleotide 3'-phosphodiesterase in isolated myelin. The ability of Triton X-100 and Sulfo betaine DLH to solubilize the enzyme was also examined. Solubilization with Triton X-100 was only effective in the presence of salt, for example with NaCl 51 % of the activity was solubilized. A single extraction with Sulfo betaine DLH yielded slightly more solubilized enzyme and did not require added salt. Both activation and solubilization of 2':3'-cyclic nucleotide 3'-phosphodiesterase appeared to be similarly dependent on detergent concentration, suggesting a common action of the detergent in the two processes. Myelin basic protein was solubilized more readily than the enzyme. In contrast with the enzyme in myelin, 2':3'-cyclic nucleotide 3'-phosphodiesterase activity in C₆ cells was not increased in the presence of Triton X-100, and was partially solubilized by either Triton X-100 or NaCl alone. No myelin basic protein could be detected in C₆ cells by radioimmunoassay.

The ability of treatment with detergents or sonication to cause an increase in the measured activity of CNPase (EC 3.1.4.37) in myelin or tissue homogenates was recognized in early studies (Kurihara & Tsukada, 1967; Kurihara *et al.*, 1969; Agrawal *et al.*, 1970; Gregson & Oxberry, 1972). Consequently most investigations involving the enzyme have utilized some form of 'activation' before assay to obtain higher and more reproducible results. Lees *et al.* (1974) described in more detail the activating effects of several detergents on CNPase in isolated myelin. This was contrasted with CNPase in erythrocyte plasma membranes, which was not activated by treatment with any of the detergents. The ability of detergents to solubilize CNPase from myelin has not been previously reported and such information would be important in devising purification procedures.

The problem of determining when material is 'solubilized' has been discussed in recent reviews (Steck & Fox, 1972; Maddy & Dunn, 1976). Membrane solubilization rarely results in the production of mixtures of completely independent molecules and solubilized material is normally defined in terms of a procedure relevant to the study being undertaken. In all the experiments reported in the present paper, soluble material has been defined as that which remains in solution after centrifugation for 1 h at greater than 100 000g. Milder centrifugation

conditions may lead to a failure to adequately sediment membrane subunits.

The activation and solubilization of CNPase in myelin and in C₆ cells were compared. The C₆ cell line was originally prepared from a chemically induced astrocytoma of rat brain (Benda *et al.*, 1968) and was found to produce high activities of CNPase (Zanetta *et al.*, 1972; Volpe *et al.*, 1975). It has been claimed that these cells also produce myelin basic protein (Volpe *et al.*, 1975) and have been shown to contain S-100 protein, a brain-specific protein normally associated with astroglial cells (Benda *et al.*, 1968). This cell would therefore appear to exhibit features of both types of glial cell and may resemble a glial precursor (glioblast).

Experimental

Materials

Triton X-100 { α -[4-(1,1,3,3-tetramethylbutyl)-phenyl]- ω -hydroxypoly(oxy-1,2-ethanediyl)} was obtained from Robert Bryce & Co., Melbourne, Australia, Sulfo betaine DLH (dodecyl ammonium sulphonic acid betaine) was from Textilana Corp., Hawthorne, CA, U.S.A., and other detergents were from BDH, Poole, Dorset, U.K. Bovine serum albumin and adenosine 2':3'-cyclic monophosphate (sodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Basic protein for use as a standard in radioimmunoassay was prepared from bovine brain by the method of Dunkley & Carnegie (1974).

Abbreviation used: CNPase, 2':3'-cyclic nucleotide 3'-phosphodiesterase.

C₆ cells were obtained from Flow Laboratories, Sydney, Australia.

Preparation of myelin

As it was hoped to obtain soluble material suitable for further purification of CNPase, the method chosen for myelin preparation was a rapid one suitable for use on a large scale (Mokrasch, 1971). Bovine spinal cord was obtained fresh from the abattoirs and immediately placed on ice for transport to the laboratory. All subsequent manipulations were performed on ice. Meninges and blood vessels were stripped from the cord and 1 g slices were removed, chopped finely with a razor blade and homogenized in 15 ml of cold 0.32 M-sucrose by using eight up-and-down strokes of a Potter-Elvehjem Teflon/glass homogenizer (0.10–0.15 mm clearance) attached to a mechanical drive unit. The homogenate was layered on 30 ml of cold 0.8 M-sucrose and centrifuged at 17500 rev./min (37000 g) for 15 min at 4°C (SS34 rotor, Sorvall RC2B centrifuge). The clear upper layer was discarded and the pinkish interface removed with a Pasteur pipette and the myelin from several centrifugations was pooled. The pooled fraction was added to 2 vol. of cold water, mixed and centrifuged at 12500 rev./min (25000 g) for 25 min (GSA rotor, Sorvall RC2B centrifuge). The pellet obtained was resuspended in cold water and centrifuged twice as above. In addition, more highly purified myelin was prepared from bovine spinal cord by the method of Norton & Poduslo (1973). All myelin was stored frozen at –20°C until required.

Activation of CNPase in myelin

Rapidly prepared myelin was diluted with cold water to provide a protein concentration of approx. 0.5 mg/ml and 0.1 ml was added to 0.05 ml of 0.05 M-Tris/HCl (pH 7.5) and 0.1 ml of detergent in the range 0–5 g/100 ml. All tubes were mixed by inversion and left on ice for 10 min. Sufficient water was then added so that CNPase activity could be determined on 0.1 ml portions without further dilution.

Examination of solubilization

In determining the solubilizing effect under various conditions, 0.1 ml of myelin (suspended in water, approx. 20 mg of protein/ml) was added to 2.4 ml of solubilizing medium in a 10 ml polypropylene centrifuge tube, and, unless otherwise specified, was mixed by inverting 4 times. Tubes were kept in an ice bath at all times. In all cases the solubilizing medium contained imidazole/HCl (pH 6.2) with a final value for *I* of 0.0125. Additives to be tested in the solubilizing medium were adjusted to concentrations such that after addition of the myelin the required concentration was attained. Approx. 2.2 ml of the myelin suspensions was placed in cellulose nitrate centrifuge tubes (7.9 mm × 49.2 mm) and with delrin

adaptors were centrifuged at 40000 rev./min (113000 g, 50 Ti rotor, Beckman ultracentrifuge) for 1 h at 4°C. Supernatants were removed with a Pasteur pipette for assay and if required for further treatment or assays the pellets were resuspended. Material not centrifuged (approx. 0.3 ml) was retained on ice and used for analysis of total activities of CNPase or other components under investigation.

C₆ cells

These were cultured in Ham's F10 medium containing 15% horse serum and 2.5% foetal calf serum, 60 units of penicillin/ml and 50 mg of streptomycin/ml and buffered with 15 mM-NaHCO₃ (pH 7.3). Cell cultures were maintained in an incubator at 37°C and gassed with CO₂/air (1:19, v/v). Harvesting was performed by using 0.14 M-NaCl/3.5 mM-Tris/HCl (pH 7.5) containing 0.02 g of EDTA/100 ml (Volpe & Marasa, 1975). Cells were resuspended in phosphate-buffered saline (Cohen *et al.*, 1973). Where sonication was used to disrupt cells this was performed on ice for 1 min at 21 kHz with a 3.2 mm probe in an MSE sonicator.

Assays

CNPase and protein were assayed as described by Sims & Carnegie (1976) and Lees & Paxman (1974) respectively. Basic protein was determined by radioimmunoassay (Cohen *et al.*, 1975). Assays on all samples were performed at least in duplicate.

Results

Activation

The influence of four detergents, Triton X-100, sodium dodecyl sulphate, sodium deoxycholate and Sulfobetaine DLH, on CNPase activity in myelin is shown in Fig. 1. Marked activation was found in all cases except with sodium dodecyl sulphate. As sodium dodecyl sulphate is well established as a protein denaturant it is not surprising that no activity was detected in the presence of high concentrations of this detergent. However, at low concentrations of sodium dodecyl sulphate an activation of the CNPase was observed.

Solubilization with Triton X-100

For most of the studies on solubilization of CNPase, Triton X-100 was utilized as this detergent produced marked activation, was readily available commercially and has been widely used in similar studies of other membrane components. A less detailed examination of the solubilizing effects of Sulfobetaine DLH was undertaken to provide a comparison.

Initially Triton X-100 was added in the presence of 0.05 M-imidazole/HCl (pH 6.2) or 0.01 M-Tris/HCl (pH 7.5) and in both cases less than 5% of the total

Fig. 1. Activation of CNPase by detergent

Myelin (0.1 ml containing approx. 50 μ g of protein) was added to 0.05 ml of 0.05 M-Tris/HCl (pH 7.5) and 0.1 ml of detergent to the final concentration shown. After mixing, tubes were left for 10 min before dilution with water for CNPase and protein determination. One unit of enzyme activity is equal to 1 μ mol of cyclic AMP hydrolysed/min. Symbols: $\bullet$, Sulfofetaine DLH; Δ , sodium dodecyl sulphate; $\circ$, sodium deoxycholate; $\square$, Triton X-100.

Fig. 2 Effect of I on Triton X-100 solubilization from myelin

Myelin (0.1 ml containing approx. 2 mg of protein) was added to 2.4 ml of solubilizing medium containing Triton X-100 (2 g/100 ml), imidazole/HCl (pH 6.2; I 0.0125) and NaCl to adjust the final I to the values shown. Solubilized material was obtained by centrifugation at 113 000 g for 1 h. Other details were as described in the Experimental section. Results shown represent pooled data from four separate experiments. All points were determined in at least two independent experiments and where appropriate the standard error is shown with the number of

Fig. 3. Effect of Triton X-100 concentration on solubilization of CNPase

Myelin (0.1 ml containing approx. 2 mg of protein) was added to 2.4 ml of solubilizing medium containing imidazole/HCl (pH 6.2; I 0.0125), NaCl to a final I of 0.75 and Triton X-100 to the final concentrations indicated. Solubilized fraction was obtained by centrifugation at 113 000 g for 1 h and other details are described in the Experimental section. Square symbols indicate total CNPase activity in uncentrifuged mixtures and the circles show the solubilized CNPase activity. Results represent pooled data for five independent experiments and points determined in more than one experiment are shown as solid symbols. Standard error is included where appropriate with the number of experiments in parentheses.

activity was recovered in the supernatant. Since I might influence the solubilizing ability of Triton X-100 the effect of adjusting I with NaCl at a constant concentration of Triton X-100 (2 g/100 ml) was studied (Fig. 2). A maximum level of approx. 50% solubilization was attained at I exceeding 0.5. The activity determined for the uncentrifuged samples was the same in all cases, i.e. the activating effect of Triton X-100 was unaffected by the I of the solubilizing medium. At high I , in the absence of Triton X-100, less than 2% of the CNPase activity was solubilized.

In Fig. 3 the effect of increasing the Triton X-100 concentration at constant I is shown. Curves are presented showing the activity both in the uncentrifuged samples and in supernatants. Activation (i.e. the enzyme activity in the uncentrifuged samples) and solubilization were both affected similarly by Triton X-100 and show a plateau at concentrations

experiments in parentheses. Activities are expressed relative to those of the uncentrifuged samples, which had a specific activity of 12.0 μ mol of AMP hydrolysed/min per mg of protein.

above 0.2 g/100 ml and a discontinuity between 1 and 2 g/100 ml. Increasing Triton X-100 concentration of the supernatants to greater than 2.5 g/100 ml after extraction did not affect the activity measured. The activation curve shown in Fig. 2 differs in shape from that in Fig. 1 and this is probably due to a difference in the Triton X-100/myelin ratio.

Thus under optimal conditions, approx. 51% of total CNPase activity was solubilized. All remaining activity was detected in the pellet, suggesting that there was no denaturation during the solubilization process. When myelin was extracted 3 times with the optimal solubilizing medium, a total of 66% of the CNPase activity was solubilized, the final extraction yielding less than 2.5% of the activity. After the third extraction the pellet was extremely difficult to resuspend and although activity remained, no accurate measure of this could be obtained. When highly purified myelin was substituted for the rapidly prepared myelin 50.3% of the total activity was obtained in a single extraction.

Figs. 4 and 5 demonstrate the effects of *I* and Triton X-100 concentration on the solubilization of the total protein and basic protein from bovine myelin. For basic protein, difficulty was experienced in obtaining sufficiently reproducible measurements

of the concentration in uncentrifuged samples. This probably was a reflection of the large dilutions required for the radioimmunoassay, which was difficult with the semi-particulate material. Thus results have been presented as absolute values rather than as percentages of the total. From the estimates obtained for uncentrifuged samples, the plateau of solubilization at high Triton X-100 and salt concentrations would represent between 65 and 100% of the total basic protein. For both the basic protein and total protein a dependence on the *I* and Triton X-100 concentration was observed that was similar to that for CNPase. However, some material was extracted at low Triton X-100 concentration (high *I*) and also at low *I* (high Triton X-100), situations where virtually no CNPase was solubilized. The plateau concentrations reached in both cases were also higher than obtained with CNPase, and the increase in extraction of basic protein with increasing Triton X-100 was less marked than was observed with CNPase.

Table 1 shows the results of modifying the extraction conditions. Increasing the severity of extraction, by using either homogenization or stirring, produced no change in the solubilization. The ability of chaotropic ions to solubilize membrane proteins is well established and for this reason two chaotropes were examined in the presence of Triton

Fig. 4. Solubilization of total myelin protein. Experimental details are as described in the legends to Figs. 2 and 3 and in the Experimental section. (a) Effect of *I* at constant Triton X-100 concentration (2 g/100 ml). (b) Effect of Triton X-100 concentration at constant *I* (0.75). All points are averaged results for two independent experiments.

Fig. 5. Solubilization of myelin basic protein. Experimental details are as described in the legends to Figs. 2 and 3 and in the Experimental section. (a) Effect of *I* at constant Triton X-100 concentration (2 g/100 ml). (b) Effect of Triton X-100 concentration at constant *I* (0.75). All points are averaged results for two independent experiments.

X-100. Guanidinium chloride in place of NaCl improved the solubilization, whereas NaSCN resulted in a decrease in solubilization. Both these salts had little effect on CNPase when used in the absence of Triton X-100. Interestingly the use of NaCl in conjunction with guanidinium chloride (in the presence of Triton X-100) produced solubilization only equivalent to that with Triton X-100 and NaCl alone.

Despite the marked dependence of solubilization on Triton X-100 concentration at concentrations less than 0.1 g/100ml, increasing the myelin content up to 5-fold at a Triton X-100 concentration of 0.1 g/100ml produced only small changes (Table 1). These results were uncorrected for the changes in volume of the supernatants due to the different sizes of the pellets of unextracted material. However, as the volume of supernatant was severalfold greater than that of the pellet, this would only mean small

differences in the percentage of CNPase solubilized from the value actually given. Thus the ratio of myelin to Triton X-100 did not appear to be a major factor in the solubilization.

Although both Triton X-100 and high *I* were required for solubilization, it was possible that the continued presence of these components was not required to maintain the CNPase in solution. Dialysis (2ml against 2×100ml of buffer for 36h) was used to selectively remove the NaCl and thus decrease the *I*. Precipitates formed in all fractions after dialysis. When this was resuspended and samples assayed, all contained 85% or higher of the original activity indicating little activity was denatured by this process. After removal of the precipitate, activity was decreased to 36% of the total solubilized in the sample dialysed against buffer containing no NaCl (*I* 0.0125). The activity was decreased to 52 and 72% where the dialysis buffer had an *I* of 0.1 and 0.2 respectively.

It remained unclear whether Triton X-100 was required for solubilization or was merely acting to remove lipid and other detergent-soluble material. Therefore attempts were made to solubilize CNPase from pellets obtained after initial extraction with Triton X-100 at low *I* (0.0125) to determine whether subsequent extractions in detergent-free buffers would solubilize CNPase (Table 2). The use of high NaCl (*I* 0.75) and a detergent-free buffer, employed for extraction of CNPase from an acetone-dried powder of brain (Guha & Moore, 1975), solubilized some activity. However, this was decreased to around 10% in both cases if the pellet was first washed with water, presumably because of a decrease in Triton X-100 associated with this pellet. As may have been expected, solubilization of around 50% was achieved when the standard conditions involving high Triton X-100 (5%) and high *I* (0.75) were employed for re-extraction.

Assay of CNPase and myelin basic protein in C₆ cells

As a comparison with the effects on myelin-bound CNPase, the effects of a variety of disruptive treatments on this enzyme in C₆ cells were examined. No CNPase activity was detected in the medium in which the cells had been growing for 4 days, and the activity in untreated cells was low (0.08 unit/mg of protein). At least 95% of the cells remained viable during the period of assay in the imidazole buffer. Cells disrupted by freezing and thawing or by sonication contained similar total activity (0.3 unit/mg of protein). Higher activity (4-fold) could be obtained by growing the cells in Dulbecco's modified Eagle's medium (pH 7.4), but the relative activities of CNPase in the soluble fraction remained the same. The proportion of CNPase activity obtained in the soluble fraction is shown in Table 3. The

Table 1. *Effect on solubilization of modification of treatment*
Summary of attempts to improve solubilization of CNPase from myelin by modifications to standard conditions. Standard conditions refers to treatment of 0.1 ml (2mg of protein) of myelin with 2.4 ml of extraction mixture with fixed concentration of 5g of Triton X-100/100ml and an *I* of 0.75 (adjusted with NaCl). Samples were mixed by inversion for 10s. Solubilized fraction was obtained by centrifugation at 113000g for 1 h.

Modification	CNPase solubilized (%)
Nil (standard conditions)	51.4
Severity of extraction*	
Homogenization (6 strokes)	52.4
Homogenization (20 strokes)	47.4
Stirring for 5–120 min	50.5 (range 47.8–53.9)
Extraction medium†	
0.5M-NaSCN	0
1M-Guanidinium chloride	3.4
2g of Triton X-100/100ml+	34.9
0.5M-NaSCN	
5g of Triton X-100/100ml+	71.5
1M-guanidinium chloride	
5g of Triton X-100/100ml+	51.3
1M-guanidinium chloride+	
0.75M-NaCl	
Triton X-100/myelin ratio‡	
0.1 ml of myelin (standard ratio)	41.2
0.2 ml of myelin	40.9
0.5 ml of myelin	33.5

* Extraction media retained as for standard conditions, but the severity of mixing altered. Homogenization was with a Potter–Elvehjem Teflon/glass homogenizer.

† All extraction media contained imidazole/HCl (pH 6.2, *I* 0.0125).

‡ Triton X-100 and *I* adjusted to 0.1 g/100ml and 0.75 respectively after addition of myelin.

Table 2. *Solubilization of CNPase after initial treatment with Triton X-100 at low I*

Myelin (0.1 ml containing approx. 2mg of protein) was added to 2.4 ml of imidazole/HCl (pH 6.2; *I* 0.0125) containing Triton X-100 at a final concentration of 5g/100ml and centrifuged at 113000 *g* for 1 h. The pellet was re-extracted with the indicated medium and recentrifuged and solubilized activity determined. A small proportion (<5%) of CNPase activity was lost in the initial extraction in all cases and results are presented as a percentage of the activity remaining in the pellet after initial extraction.

Re-extraction medium	CNPase solubilized (%)
No washing between extractions	
5% Triton X-100, imidazole/HCl, NaCl (pH 6.2; <i>I</i> 0.75)	47.4
Imidazole/HCl (pH 6.2; <i>I</i> 0.0125)	0
Imidazole/HCl, NaCl (pH 6.2; <i>I</i> 0.75)	22.5
0.05 M-4-Morpholine-ethanesulphonic acid (pH 6.0); 10 ⁻⁴ M-dithiothreitol and EDTA*	0
0.2 M-4-Morpholine-ethanesulphonic acid (pH 6.0); 10 ⁻³ M-dithiothreitol and EDTA	0
0.2 M-4-Morpholine-ethanesulphonic acid (pH 6.0); 10 ⁻³ M-dithiothreitol and EDTA + 1 M-guanidinium chloride*	18.6
2× Water wash of pellet before re-extraction	
Imidazole/HCl, NaCl (pH 6.2; <i>I</i> 0.75)	7.9
0.2 M-4-Morpholine-ethanesulphonic acid (pH 6.0); 10 ⁻³ M-dithiothreitol and EDTA + 1 M-guanidinium chloride*	12.2

* Media used by Guha & Moore (1975) for extraction of CNPase from acetone-dried powders.

Table 3. *Solubilization of CNPase from C₆ cells*

C₆ cells (1.5×10⁵ cells) suspended in phosphate-buffered saline (100 μl) were treated as indicated and activity was determined in supernatants obtained after centrifugation at 113000 *g* for 1 h.

Treatment	CNPase in supernatant (%)
Sonication (in phosphate-buffered saline)*	28.4
Freezing and thawing (in phosphate-buffered saline)*	25.5
Triton X-100 (5g/100ml)†	45.6
<i>I</i> 0.75 (adjusted with NaCl)†	40.0
Triton X-100 (5g/100ml); <i>I</i> 0.75 (adjusted with NaCl)†	75.2

* Sonication with a 3.2mm probe, 21 kHz for 1 min; freezing and thawing was carried out 3 times rapidly.

† These solutions contained imidazole/HCl (pH 6.2; *I* 0.0125). Cells were centrifuged at 1000 *g* for 5 min to remove phosphate-buffered saline and resuspended in the buffer described.

presence of activity in the soluble fraction after mild disruptive treatment has been reported previously (Volpe *et al.*, 1975) and the value of 26% obtained in the present paper for frozen and thawed material is similar to their result of 18%. The proportion of soluble CNPase was virtually unaffected by the use of sonication for cell disruption. Soluble activity was, however, markedly affected by the other

Fig. 6. *Solubilization of CNPase by Sulfobetaine DLH* Myelin (0.1 ml containing approx. 2mg of protein) was added to solubilizing medium containing imidazole/HCl (pH 6.2; *I* 0.0125) and Sulfobetaine DLH to the final concentrations shown. Solubilized fractions were obtained after centrifugation at 113000 *g* for 1 h. Other details are described in the Experimental section. Symbols: ■, total measured CNPase; ●, solubilized CNPase activity. All points represent the average results from two independent experiments. Activities are expressed relative to those of uncentrifuged samples containing 7g of detergent/100ml, which had a specific activity of 13.4 μmol of AMP hydrolysed/min per mg of protein.

treatments, the maximum effect being achieved with Triton X-100 at high *I* (i.e. the treatment that solubilized 51% of myelin activity). Although pro-

ducing some solubilization, Triton X-100 had no activating effect on the CNPase activity measured in C₆ cells.

Volpe *et al.* (1975) claimed from electrophoretic evidence that myelin basic protein was present in C₆ cells. Despite varying media, growth conditions, detergents, etc., and sources of C₆ cells, it was not possible to detect myelin basic protein in these cells by radioimmunoassay (L. B. Horvath & P. R. Carnegie, unpublished work; McDermott & Smith, 1978). Rat myelin basic protein, when added to a C₆-cell culture, could readily be measured by the radioimmunoassay.

Solubilization with Sulfobetaine DLH

Fig. 6 shows the effect of Sulfobetaine DLH on the solubilization of CNPase from bovine myelin and also shows the activation effect of the detergent under these conditions. As with Triton X-100 both activation and solubilization of CNPase were similarly affected by the detergent. The amount of activity solubilized by Sulfobetaine DLH was slightly higher than that with Triton X-100, especially when account is taken of the fact that the total activity measurable was higher in Sulfobetaine DLH than in Triton X-100.

As with Triton X-100, attempts were made to modify the extraction by using more vigorous treatments such as homogenization and stirring (up to 30 min). When homogenization was performed for 20 strokes with the Teflon/glass homogenizer, the CNPase solubilized was decreased to 49% of the total, whereas with all other treatments solubilization remained constant at around 58%. This decrease was presumably due to denaturation of the enzyme under these conditions.

Discussion

In the last decade models proposed for membrane structure have changed extensively. Part of the impetus for the new models arose from a need to account for the finding on the limited accessibility and solubility of some membrane proteins. Thus in the lipid-globular-protein-mosaic model (Singer, 1972) proteins are broadly categorized as peripheral or integral on the basis of the ease with which they are solubilized from the membrane. Peripheral proteins are those that are located at the membrane surface and are readily extracted by salts or acids. Integral proteins are those embedded (at least partially) within the membrane, and are able to be solubilized only after the membrane structure has been destroyed with organic solvents or detergents. Although the complete applicability of this model to myelin is questionable, the requirements for both detergent and the salt for solubilization of CNPase clearly places this in a category equivalent to the

intrinsic proteins. Isolation and subsequent purification of CNPase without the need for detergents has been reported (Guha & Moore, 1975; Drummond *et al.*, 1978). The starting material for this was a powder prepared by treatment of whole brain with the organic solvents acetone, butanol and light petroleum (b.p. 30–60°C). Extraction of the enzyme was then achieved by using buffered guanidinium chloride containing dithiothreitol and EDTA. When this extraction buffer was tested on myelin pretreated with Triton X-100, little solubilization of CNPase was achieved (Table 2).

It has been demonstrated in a number of studies that Triton X-100 has little effect on protein-protein interaction (reviewed by Helenius & Simons, 1975). The requirement for salt as well as Triton X-100 to produce solubilization of CNPase suggests a need to disrupt ionic interactions and it is likely that some of these are protein-protein in nature. Whether CNPase is involved in such interactions under normal circumstances in myelin or whether these are induced by the removal of material by Triton X-100 cannot be determined. Despite attempts to increase the severity of treatment of the myelin, the maximum solubilization achieved in a single extraction, when NaCl was used for adjustment of *I*, was 51%. The inability to achieve a higher value than this probably resulted from involvement of CNPase in interaction with a number of components, only some of these interactions being disrupted by the higher *I*. However, the possibility must be considered that CNPase has several forms in myelin that are differentially affected by the extraction procedure. The discontinuity observed when Triton X-100 concentration was varied would support this possibility (Fig. 3). The observation that guanidinium chloride improved the extraction, whereas NaSCN diminished it, indicates that the solubilization achieved is a function of the ability of the salt to disrupt the prevailing interactions. Incomplete and differential effects of salts on membrane proteins have also been found in a number of other studies (Garewal & Wasserman, 1974; Hatefi & Hanstein, 1974).

Useful information may be obtained from the comparison of solubilization and activation measured under the same conditions. Both processes show a similar dependence on Triton X-100 concentration at high *I* (Fig. 2), suggesting that the mechanism for the two may involve common features. However, solubilization does not necessarily occur when activation is achieved. Thus, whereas the activating effect of Triton X-100 was independent of *I*, solubilization was markedly dependent on it, and at low *I* maximal activation was achieved in the virtual absence of solubilization. Two possible models arise to explain the observed activation of CNPase. Either the Triton X-100 may act directly on the enzyme, presumably through conformational

changes, or the apparent activation occurs because accessibility of the substrate to active sites increases as Triton X-100-soluble material is removed from the myelin. From the experiments performed in the present paper neither of these mechanisms can be eliminated. However, it was demonstrated that increasing the Triton X-100 concentration of supernatants obtained at sub-optimal concentrations of Triton X-100 did not increase the activity in the supernatant. Thus for this result to be consistent with the first model it would be necessary to propose that only CNPase, which was maximally activated by Triton X-100, was present in the soluble fraction. Furthermore, this model provides no explanation for the similarity of dependence of solubilization and activation on Triton X-100. Therefore, the second model, suggesting activation through increased exposure, is favoured and this is supported by several other pieces of evidence. Kurihara & Tsukada (1967) demonstrated that some activation was achieved with sonication, a procedure that would disrupt the myelin, but would be unlikely to affect the CNPase directly. Furthermore, as shown in Fig. 1, a number of detergents from different classes produced activation of the CNPase and it seems more likely that this was the result of disruption of myelin rather than a similar effect of each of these detergents directly on the CNPase. In these studies, and in all studies where activation was routinely used before assay, samples were diluted in water after detergent treatment without affecting the activation, suggesting that once disruption was achieved, continued presence of detergent was not required. Finally Triton X-100 had no effect on CNPase powders prepared from bovine white matter by extraction with acetone, butanol and light petroleum (b.p. 30–60°C), procedures that extensively delipidated the white matter (Guha & Moore, 1975). Thus it seems highly likely that activation results from increased exposure of CNPase active sites to substrate. Lees *et al.* (1974) came to a similar conclusion in their study of activation of CNPase. The similarity of the dependence of solubilization and of activation on Triton X-100 probably arises because the increased accessibility of substrate to active sites would be paralleled by increased exposure of the enzyme molecules to the effect of salts, thus increasing the solubilization. By comparison with Triton X-100, the solubilization produced by Sulfobetaine DLH showed no requirements for added salt. As this zwitterionic detergent carries both positively and negatively charged groups it would appear to fill the role of both Triton X-100 and NaCl in the extraction process.

Solubilization of total myelin protein and myelin basic protein showed a similar dependence on the *I* and Triton X-100 concentrations to that observed for CNPase. However, differences did occur in the

maximum proportion of material solubilized and the amounts of solubilization achieved when either Triton X-100 or NaCl was absent. Interestingly, some basic protein was solubilized by salt alone, whereas the majority was only extracted with both salt and Triton X-100. Myelin basic protein is modified *in vivo* by methylation, phosphorylation and deamidation resulting in an array of protein molecules differing only at a few amino acid residues (Chou *et al.*, 1977; Martenson *et al.*, 1976). It is likely that such modifications will affect the relationship between basic protein and other myelin components and the differential solubilization of some basic protein under comparatively mild conditions may reflect differences in the localization of the various forms of the protein.

Determination of CNPase activity in 113000g supernatants from C₆ cells after various treatments indicated that CNPase was far more readily extracted from this material than from myelin. The proportion of enzyme solubilized from the particulate fraction of the cells may be determined by excluding from the calculations the amount of CNPase found in the soluble fraction after mild disruptive treatment such as sonication (Table 3). Thus CNPase solubilized from the particulate fraction was 18% with buffered NaCl, 25% with buffered Triton X-100 and 66% with buffered Triton X-100/NaCl mixtures. With all three conditions the solubilization was higher than that obtained from myelin with the corresponding extraction medium. Thus it would seem that the enzyme is far more accessible in these cells than in myelin. Furthermore the lack of an activating effect of Triton X-100 would suggest that the active sites are completely exposed to substrate once the cell is lysed. It would be of interest to determine whether the same is true of oligodendroglial cells in view of the widely accepted proposal for myelin formation involving transition from oligodendroglial plasma membrane. The failure to demonstrate myelin basic protein in the C₆ cells by radioimmunoassay renders these cells less useful as a neoplastic counterpart of the oligodendroglial cell (McDermott & Smith, 1978).

In conclusion, partial solubilization of CNPase in isolated myelin has been achieved by extraction with detergents. Solubilization and activation of CNPase were both dependent on the ability of the detergent to disrupt the myelin. Solubilization was unaffected by the severity of the extraction procedure.

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