Phosphorylation *in vivo* of four basic proteins of rat brain myelin

Harish C. AGRAWAL, Keith O'CONNELL, Charlotte L. RANDLE and Daya AGRAWAL
Departments of Pediatrics and Neurology, Washington University School of Medicine, 500 South
Kingshighway, P.O. Box 14871, St. Louis, MO 63178, U.S.A.

(Received 14 May 1981/Accepted 16 September 1981)

When rat brain myelin was examined by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis followed by fluorography of the stained gel, it was found that a host of proteins of rat brain myelin were labelled 2, 4 and 24 h after the intracerebral injection of $H_3^{32}PO_4$. Among those labelled were proteins migrating to the positions of myelin-associated glycoprotein, Wolfgram proteins, proteolipid protein, DM-20 and basic proteins. The four basic proteins with mol.wts. 21 000, 18 000 (large basic protein), 17 000 and 14 000 (small basic protein) were shown to be phosphorylated after electrophoresis in both acid-urea- and sodium dodecyl sulphate-containing gel systems followed by fluorography. The four basic proteins imparted bluish-green colour, after staining with Amido Black, which is characteristic of myelin basic proteins. The four basic proteins were purified to homogeneity. Fluorography of the purified basic proteins after re-electrophoresis revealed the presence of phosphorylated high-molecular-weight 'polymers' associated with each basic protein. The amino acid compositions of the phosphorylated large basic protein and small basic proteins are compatible with the amino acid sequences. Proteins with mol.wts. 21 000 and 17 000 gave the expected amino acid composition of myelin basic proteins. Radiolabelled phosphoserine and phosphothreonine were identified after partial acid hydrolysis of the four purified basic proteins. The [^{32}P]phosphate-protein bond in the basic protein was stable at an acidic pH but was readily hydrolysed at alkaline pH, as would be expected of phosphoester bonds involving both serine and threonine residues. Double-immunodiffusion analysis demonstrated that the four phosphorylated proteins showed complete homology when diffused against antiserum to a mixture of small and large basic proteins. Since the four basic proteins of rat brain myelin were phosphorylated both *in vivo* and *in vitro* it is postulated that the same protein kinase is responsible for their phosphorylation in both conditions.

Myelin isolated from most vertebrate central nervous systems contains only large basic protein with a molecular weight of 18 400. The rat and mouse brain contain in addition a smaller basic protein with a molecular weight of 14 000 (Mehl & Halaris, 1970; Martenson *et al.*, 1971; Agrawal *et al.*, 1972). Allison *et al.* (1974) first described the presence of another basic protein with a molecular weight of 17 000 in rat brain myelin. Subsequently Barbarese *et al.* (1978) provided biochemical and immunochemical evidence of the presence of two subsidiary basic proteins with molecular weights of 21 500 and 17 500 in mouse brain myelin. A number of

investigators have demonstrated the presence of soluble, membrane-bound and Ca^{2+} -stimulated protein kinases in central-nervous-system myelin that phosphorylate small and large basic proteins *in vitro* and *in vivo* (Carnegie *et al.*, 1973, 1974; Steck & Appel, 1974; Miyamoto & Kakiuchi, 1974, 1975; Miyamoto, 1975, 1976; McNamara & Appel, 1977; Matthieu & Kuffer, 1978; Petrali *et al.*, 1980; Sulakhe *et al.*, 1980). The phosphorylation of other proteins in myelin has not been reported. We present evidence that a host of proteins associated with the isolated preparations of rat brain myelin were labelled 2, 4 and 24 h after intracerebral injection of [^{32}P]P_i. The four phosphorylated basic proteins with molecular weights of 21 000, 18 000 (large basic protein), 17 000 and 14 000 (small basic protein) were purified and characterized.

Abbreviations used: MDPF, 2-methoxy-2,4-diphenyl-furan-3(2*H*)-one; SDS, sodium dodecyl sulphate.

Materials and methods

Chemicals

All reagents used for SDS/polyacrylamide-gel electrophoresis were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and Bio-Rad (Richmond, CA, U.S.A.). MDPF was obtained from Hoffman-LaRoche (Nutley, NJ, U.S.A.). Carrier-free $\text{H}_3^{32}\text{PO}_4$ was obtained from New England Nuclear (Boston, MA, U.S.A.). Instafluor, Soluene-350 and 2,5-diphenyloxazole were purchased from Packard Instrument Co., IL, U.S.A. Developing rats (28–32 days old) were lightly anaesthetized with diethyl ether and were injected intracerebrally ($40\mu\text{l}$) with 1mCi of $\text{H}_3^{32}\text{PO}_4$ using a 0.5ml sterile insulin syringe. The animals were killed by decapitation 2, 4 or 24 h later. The average body and brain weights of 28–32-day-old rats were $88 \pm 0.95\text{g}$ and $1.7 \pm 0.05\text{g}$ (means $\pm$ S.E.M.) respectively.

Isolation of myelin

All operations were carried out at $0-4^\circ\text{C}$. A 5% (w/v) homogenate of fresh rat brain was prepared in 0.88M -sucrose and myelin was isolated as described by Agrawal *et al.* (1972). Purified myelin was suspended in water and immediately freeze-dried. Dry myelin was stored over Drierite at -80°C . The yield of dry myelin was $29.9 \pm 1.45\text{mg}/\text{brain}$ (mean $\pm$ S.E.M.).

Delipidation of myelin proteins

Lipids were removed from dry myelin (25mg) by successive extraction with diethyl ether/ethanol (3:2, v/v) and diethyl ether as described by Agrawal *et al.* (1974). Myelin proteins were dissolved in a sample buffer containing 10mM -borate buffer, pH 9.0, containing 1% sodium dodecyl sulphate and 1.5% dithiothreitol, at a concentration of 2mg of protein/ml. This solution was heated in a boiling-water bath for 2 min and stored in $50\mu\text{l}$ portions at -80°C .

Preparation of basic protein-enriched fractions

All operations were carried out at $0-4^\circ\text{C}$. Basic proteins were isolated from delipidated myelin (200mg) by the technique of Martenson *et al.* (1969) and Agrawal *et al.* (1972). The yield of basic proteins by this method was 50% of the expected value. Basic proteins were also isolated from dry myelin (170mg) by the procedures of Gonzales-Sastre (1970) as modified by Nussbaum *et al.* (1974). The pellets containing basic proteins were treated four times with 0.03M -HCl followed by centrifugation at 18000g for 30 min. The four supernatants were combined, filtered, dialysed against 20 vol. of 0.1M -acetic acid and were freeze-dried. The freeze-dried basic proteins were reconstituted in ice-cold water, and centrifuged at 37000g for 1 h. The protein concentration in the supernatant

was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The recovery of basic protein was 60–70% of the expected value. The radioactivity was determined after adding 10ml of 3a70 solution in a Packard liquid-scintillation spectrometer maintained at 4°C . The counting efficiency was 90% as determined by internal standardization.

A substantial amount of lipids and faster-moving protein components were associated with myelin basic proteins, regardless of the method of isolation. However, basic proteins prepared by both methods gave identical protein profiles.

SDS/polyacrylamide-slab-gel electrophoresis and fluorography

Myelin proteins were separated by SDS/polyacrylamide-slab-gel electrophoresis by using the discontinuous buffer system described by Neville (1971). The running gel ($13\text{cm} \times 12\text{cm} \times 1.5\text{mm}$) contained 12% (w/v) acrylamide, 0.32% (w/v) bisacrylamide, 0.8488M -Tris/HCl (pH 9.18), and was polymerized by the addition of $\text{NNN}'\text{N}'$ -tetramethylethylenediamine (TEMED) and ammonium persulphate to final concentrations of 0.01% and 0.24% respectively. The lower concentration of TEMED in the running gel was essential to clearly separate 21000- and 17000-mol.wt. basic proteins from DM-20 (Agrawal *et al.*, 1972) and large basic protein (Allison *et al.*, 1974) respectively. The stacking gel ($1\text{cm} \times 12\text{cm} \times 1.5\text{mm}$) contained 5% (w/v) acrylamide, 0.135% (w/v) bisacrylamide, and 0.1082M -Tris/sulphate (pH 6.1). The gel was polymerized by the addition of TEMED and ammonium persulphate to a final concentration of 0.15% (v/v) and 0.04% (w/v) respectively.

The myelin proteins were dissolved in 10mM -borate buffer (pH 9.0), containing 1% SDS and 1.5% dithiothreitol and $50\mu\text{l}$ of the following samples were applied per well: total myelin proteins ($100-108\mu\text{g}$); basic protein-enriched fraction ($50-108\mu\text{g}$) and purified samples of 21000-mol.wt. protein; large basic protein; 17000-mol.wt. protein; and small basic protein ($11\mu\text{g}$). The lower- and upper-reservoir buffers were those described by Neville (1971). Electrophoresis was carried out first at 40 V for 90 min, then at 80 V for 90 min, and finally at 160 V for 3 h. The gels were stained with Coomassie Brilliant Blue, then destained as described by Agrawal *et al.* (1972). The same gel was then fluorographed at -80°C as described by Bonner & Laskey (1974).

Disc- and slab-gel electrophoresis of myelin basic proteins in acid-urea gel system

Basic protein-enriched fractions were dissolved in 8M -urea at a concentration of 2.4mg of protein/ml and examined by disc electrophoresis (Martenson &

Deibler, 1975). Running slab gels (14 cm × 12 cm × 1.5 mm) containing 10% (w/v) acrylamide were polymerized by the method of Martenson & Deibler (1975). The stacking gel (3 cm × 12 cm × 1.5 mm) contained 5% (w/v) acrylamide, 8 M-urea, 1 M-acetic acid and was polymerized by the addition of 1 ml of 12% (v/v) TEMED and 1 ml of 6.72% (w/v) ammonium persulphate per 22.1 ml of the gel mixture. It was essential to increase the concentration of both TEMED and ammonium persulphate to polymerize the stacking gel. Sample (40 µl) containing 50 µg of basic proteins and 15 µl of basic fuchsin (tracking dye) were applied to each well. Electrophoresis was carried out at 4 mA for 4 h and then at 8 mA for 18 h. The gels were stained with 1% (w/v) Amido Black in 7% acetic acid for 1 h, destained in 3% acetic acid and then fluorographed as described above.

Radioactive measurements

The stained disc gels were cut into 1 mm sections, which were heated with 1 ml of Soluene-350 at 60°C for 18 h. After the addition of 10 ml of Instafluor, radioactivity was determined as described above.

Conjugation of myelin basic proteins with MDPF and purification of 21 000-mol.wt. protein, large basic protein, 17 000-mol.wt. protein and small basic protein

Basic proteins prepared by the second method were coupled to MDPF as described by Fishman *et al.* (1979). Disc-gel electrophoresis in the presence of SDS was carried out by the procedure of Weber & Osborn (1969) with the modification introduced by Allison *et al.* (1974) to clearly separate the 21 000-mol.wt. protein and 17 000-mol.wt. protein from large basic protein. The fluorescent protein bands were excised under long-wavelength (360 nm) u.v. light and the proteins eluted with 0.1% SDS containing 0.1% 2-mercaptoethanol at 37°C until the gels were completely devoid of fluorescence. The protein eluates were freeze-dried, reconstituted in 0.1 vol. of water and dialysed against 0.1% SDS. The retentate was centrifuged at 10 000 g for 10 min. The clear supernatant was used to determine the protein concentration and radioactivity of the isolated basic proteins. The purity of the purified proteins was determined by disc- and slab-gel electrophoresis in the continuous and discontinuous SDS/buffer systems.

Separation of phosphoserine and phosphothreonine

Purified basic proteins and eluate from the blank gel were exhaustively dialysed against 0.001% SDS (four changes of 2000 ml in 24 h) and freeze-dried. These preparations were used for the separation of phosphoamino acids by high-voltage electrophoresis and amino acid analysis. ³²P-labelled 21 000-mol.wt.

protein (35 µg), large basic protein (125 µg), 17 000-mol.wt. protein (75 µg) and small basic protein (250 µg) were hydrolysed in the presence of unlabelled phosphoserine and phosphothreonine in 6 M-HCl for 4 h. The hydrolysates were dried *in vacuo*, dissolved in 0.5 M-acetic acid, and [³²P]-phosphoserine and [³²P]phosphothreonine were separated by high-voltage electrophoresis at pH 1.9 (Allerton & Pearlman, 1965). Radiolabelled phosphoamino acids were located by fluorography at -80°C after spraying the chromatogram with 7% 2,5-diphenyloxazole dissolved in diethyl ether (Randernath, 1979). The same chromatogram was subsequently sprayed with ninhydrin to stain phosphoserine and phosphothreonine (Allerton & Pearlman, 1965).

Amino acid analysis of ³²P-labelled 21 000-mol.wt. protein, large basic protein, 17 000-mol.wt. protein and small basic protein

Five samples each of large and small basic protein and two samples each of 21 000- and 17 000-mol.wt. protein were hydrolysed with 6 M-HCl *in vacuo* for 22 h, and amino acids in the hydrolysate were analysed with a Beckman 119-CL Automatic Amino Acid Analyzer equipped with an integrator.

Preparation of antiserum and immunodiffusion

Basic proteins were eluted from the acrylamide-gel sections with water (1 ml/gel section) until the gel particles were devoid of fluorescence. The eluate was centrifuged at 10 000 g for 10 min, and the clear supernatant was filtered through millipore and freeze-dried. The purified proteins were dissolved in 0.5% Triton X-100/0.1% SDS, containing 0.8% NaCl. Double-immunodiffusion analysis was carried out using antiserum to a mixture of large and small basic protein from rat brain myelin (Agrawal *et al.*, 1977; Hartman *et al.*, 1979). The precipitin line could be clearly seen in 48 h. Non-specific precipitation was controlled by diffusing (a) the eluate from the blank gel against antiserum to basic proteins and (b) the pre-immune serum against the basic proteins.

Results

SDS/polyacrylamide-slab-gel electrophoresis followed by fluorography of the stained gel revealed that at least 15 proteins of rat myelin were readily labelled 2 h and 4 h after the intracerebral injection of H₃³²PO₄ (Fig. 1).

Identification of phosphorylated basic proteins

When fractions enriched in basic proteins (prepared by methods 1 and 2) were examined by disc electrophoresis in an acid-urea gel system, they were resolved into four protein bands, but determination

Fig. 1. SDS/polyacrylamide-slab-gel electrophoresis of phosphorylated proteins of rat brain myelin

Sample (100 µg of delipidated total myelin proteins containing 280 000 c.p.m.) in 50 µl was applied to the gel. In (a), the molecular weights of the standard proteins are, from top to bottom (1–11), β-galactosidase (130 000), phosphorylase (94 000), conalbumin (76 000), catalase (60 000), ovalbumin (43 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), chymotrypsinogen (25 700), soya-bean trypsin inhibitor (21 000), myoglobin (17 200), lysozyme (13 700) and cytochrome *c* (11 700). (b) shows the protein profile stained with Coomassie Blue. (c) shows a fluorogram of gel (b). Gel was exposed to X-ray film for 4 days at –80°C. Identical results were obtained when myelin proteins from six different experiments were examined. Abbreviations used: WP, Wolfram proteins; PLP, proteolipid protein; 21K, 21 000-mol.wt. protein; LBP, large basic protein; 17K, 17 000-mol.wt. protein; SBP, small basic protein.

of [^{32}P]P_i in 1 mm gel sections of the stained gels revealed only two peaks of radioactivity, which appeared to correspond to large and small basic protein (Fig. 2). However, when these proteins were separated on an acid-urea slab gel and fluorographed, all four proteins were phosphorylated (Fig. 3b). The four basic proteins imparted a bluish-green

Fig. 2. Disc-gel electrophoresis in acid-urea gel system of phosphorylated basic proteins (100 µg of basic proteins containing 5000 c.p.m.)

Electrophoresis was carried out for 4 h at 1.5 mA/gel and the radioactivity in the stained gel was determined as described in the text. Identical results were obtained from myelin basic proteins (prepared by methods 1 and 2) in four different experiments. Note the presence of proteins migrating above the large basic protein (LBP) and the small basic protein (SBP).

colour after staining with Amido black, which is characteristic of myelin basic protein (Martenson *et al.*, 1971).

Purification and fluorography of phosphorylated basic proteins

MDPF-labelled purified basic proteins migrated as a single band when examined in disc gels in both continuous (Weber & Osborn, 1969) and discontinuous (Neville, 1971) SDS gel systems respectively, as shown in Figs. 4 and 5(a). Examination of purified basic proteins by SDS-slab-gel electrophoresis (Neville, 1971) followed by fluorography of the stained gel provided further evidence of phosphorylation of 21 000-mol.wt. protein, large basic protein, 17 000-mol.wt. protein and small basic

Fig. 3. Slab-gel electrophoresis in the acid-urea gel system of phosphorylated basic proteins

Sample ($50\mu\text{g}$ of basic protein containing 520 c.p.m.) in $40\mu\text{l}$ was applied to the gel. (a) shows proteins stained with Amido Black. (b) shows a fluorograph of gel (a). Gel was exposed to X-ray film for 17 days at -80°C . The occluded radioactivity on top of gel B is due to lipids. Protein bands 1, 2, 3 and 4 correspond to 21 000-mol.wt. protein, large basic protein, 17 000-mol.wt. protein and small basic protein respectively.

protein as shown in Fig. 5(b). It is of interest to mention that a high-molecular-weight 'polymer' was

Fig. 4. Purified proteins of rat brain myelin separated on 12% acrylamide gel

The composition of the gel and electrode buffer was 0.1 M-sodium phosphate buffer (pH 7.2) containing 0.1% (w/v) SDS. Samples containing $25\mu\text{g}$ of purified proteins and $100\mu\text{g}$ of basic protein-enriched fraction were loaded on to the disc gel. Electrophoresis was carried out first at 1 mA/gel for 1 h, 2 mA/gel for 3 h, then at 3 mA/gel for 18 h and finally at 8 mA/gel for 2 h. Gels were stained with Coomassie Blue and destained as described in the text. (1) Myelin basic proteins soluble in 0.1 M-HCl; (2) 21 000-mol.wt. protein (21 K); (3) large basic protein (LBP); (4) 17 000-mol.wt. protein (17 K); (5) small basic protein (SBP). The presence of high-molecular-weight 'polymer' is the purified preparation of four basic proteins is indicated by the arrow.

always seen in the purified preparations of each of the basic proteins (Fig. 4) and fluorography of the stained gels revealed that the 'polymers' were also phosphorylated (Fig. 5b).

When purified small basic protein was eluted from the gel and subjected to re-electrophoresis, this 'polymer' was seen after staining and fluorography of the gel (Fig. 6).

Molecular-weight determination

The molecular weights of the four purified phosphorylated basic proteins were 21 000, 18 000 (large basic protein), 17 000 and 14 000 (small basic protein; Figs. 1 and 4) as determined by SDS/polyacrylamide-gel electrophoresis (Neville, 1971; Allison *et al.*, 1974). These results are in excellent agreement with the values for four basic proteins of mouse brain myelin reported by Barbarese *et al.* (1978). The conjugation of basic proteins with MDPF did not alter their electrophoretic mobility in the SDS gel system (results not presented).

Fig. 5. *SDS/polyacrylamide-slab-gel electrophoresis of purified phosphorylated basic proteins*

(a) Proteins stained with Coomassie Blue. (b) Fluorograph. Lanes 1–6 show the same protein in (a) and (b). Lane 1, delipidated myelin proteins (108 μ g of proteins containing 3693 c.p.m.); lane 2, 21000-mol.wt. protein (21K; 11 μ g of protein containing 224 c.p.m.); lane 3, large basic protein (LBP, 11 μ g of protein containing 179 c.p.m.); lane 4, 17000-mol.wt. protein (17K; 11 μ g of protein containing 327 c.p.m.); lane 5, small basic protein (SBP; 11 μ g of protein containing 206 c.p.m.); lane 6, basic protein-enriched fraction (52 μ g of protein containing 2540 c.p.m.). The phosphorylated high-molecular-weight 'polymer' is indicated by the arrow. Other abbreviations are defined in the legend to Fig. 1.

Fig. 6. *SDS/polyacrylamide-slab-gel electrophoresis of repurified small basic protein*

(a) Proteins stained with Coomassie Blue; (b) fluorograph of the same gel. Lane (1) and (4), basic protein-enriched fraction (50 μ g; 2200 c.p.m.); lanes (2) and (5), small basic protein (SBP; 10 μ g; 180 c.p.m.); lanes (3) and (6), repurified small basic protein (SBP; 15 μ g; 250 c.p.m.). The presence of a high-molecular-weight 'polymer' is indicated by the arrow. Abbreviations used: LBP, large basic protein; SBP, small basic protein. The numbers to the left of the gels refer to the molecular weight ($\times 10^{-3}$).

Amino acid composition of ^{32}P -labelled basic proteins

The results of amino acid analysis of five samples each of large and small basic protein and two samples each of 21000- and 17000-mol.wt. protein are shown in Table 1. The results of amino acid composition of large and small basic proteins are similar to those reported by Martenson *et al.* (1972). The amino acid compositions of the 21000- and 17000-mol.wt. proteins are similar to the expected amino acid compositions of myelin basic proteins. The composition of small basic protein is compatible with the known sequence of small basic protein (Dunkley & Carnegie, 1974). The consistently lower concentration of lysine, arginine and histidine is probably due to covalent linkage of MDPF with free amino groups of basic amino acids in these proteins, because normal values for these amino acids were obtained when native small and large basic protein without conjugation with MDPF were analysed for the amino acid content.

Determination of ester linkage

Two pieces of evidence suggest that radiolabelled [^{32}P]P_i was covalently linked by phosphoester bonds to both serine and threonine residues of purified phosphorylated basic proteins. First, [^{32}P]P_i remained bound after successive treatment of purified basic proteins with trichloroacetic acid and diethyl ether, but was readily released after treatment with 1 M-NaOH (results not shown). Secondly, limited acid hydrolysis of phosphorylated basic proteins followed by separation of phosphorylated amino acids by high-voltage electrophoresis and fluorography of the chromatogram revealed that ^{32}P was associated with both phosphoserine and phosphothreonine residues of the four basic proteins (Fig. 7). Fig. 7 also shows that more threonine residues were phosphorylated or the same sites were phosphorylated to a greater extent in 17000-mol.wt. protein and small basic protein compared with 21000-mol.wt. protein and large-basic protein.

Table 1. *Amino acid composition of MDPF-conjugated ³²P-labelled basic proteins of rat brain myelin*

Five separate samples each of large basic protein (LBP) and small basic protein (SBP) and two samples each of 21 000-mol.wt. protein (21K) and 17 000-mol.wt. protein (17K) were hydrolysed and the hydrolysate was analysed in duplicate to determine the amino acid composition as described in the Materials and methods section. Tryptophan was destroyed during the acid hydrolysis of the proteins.

Amino acid	Amino acid composition (residues/mol)			
	21K	LBP	17K	SBP
Aspartic acid	14	12	13	10
Threonine	8	9	8	8
Serine	19	20	16	16
Glutamic acid	16	11	12	7
Proline	12	11	13	9
Glycine	30	24	24	15
Alanine	13	11	19	6
Valine	3	2	3	2
Methionine	3	2	2	2
Isoleucine	3	3	3	2
Leucine	10	8	9	5
Phenylalanine	8	8	6	5
Tyrosine	5	5	3	3
Histidine	9	7	8	6
Lysine	9	10	8	5
Arginine	16	15	17	14

Immunochemical studies

Immunodiffusion analysis showed that 21 000- and 17 000-mol.wt. proteins formed a line of identity with small and large basic proteins when diffused against antiserum to a mixture of large and small basic protein (Fig. 8) or antiserum to chick brain basic protein (results not shown). These observations suggest that the basic proteins share common antigenic sites, and their antigenic site(s) was not altered by coupling basic proteins with MDPF.

Time course of incorporation of ³²P into basic proteins

It is evident from Table 2 that a 50% decrease in radioactivity was observed when the basic protein-enriched fraction (prepared by methods 1 and 2) was coupled with MDPF in the presence of SDS and then dialysed against 0.1% SDS. However, there was no change in the specific radioactivity of the dialysed basic protein-enriched fraction at 2, 4 and 24 h after the administration of H₃³²PO₄. The specific radioactivities of 21 000-mol.wt. protein, large basic protein, 17 000-mol.wt. protein and small basic protein at 2, 4 and 24 h after the injection of H₃³²PO₄ are not significantly different (Table 2).

Fig. 7. *Fluorograph of [³²P]phosphoserine and [³²P]-phosphothreonine of purified basic proteins separated by high-voltage electrophoresis after partial acid hydrolysis of purified basic proteins*
(A) H₃³²PO₄; (B) hydrolysate of 21 000-mol.wt. protein (333 c.p.m.); (C) hydrolysate of large basic protein (124 c.p.m.); (D) hydrolysate of 17 000-mol.wt. protein (681 c.p.m.); (E) hydrolysate of small basic protein (280 c.p.m.); (F) a mixture of phosphoserine (5 μg) and phosphothreonine (5 μg) standards stained with ninhydrin. The chromatogram was kept in contact with X-ray film at -80°C for 30 days. Abbreviations used: IP, P; P-Ser, phosphoserine; P-Thr, phosphothreonine.

Fig. 8. *Identification of purified phosphorylated basic proteins by double-immunodiffusion analysis*
Twenty microlitres of rabbit anti-(rat brain basic protein) was placed in the centre well and 40 μl of sample was placed in each of the six peripheral wells. The Ouchterlony plates were incubated in a humid atmosphere at 4°C for 48 h. (1) Eluates from the blank gel; (2) ³²P-labelled small basic protein (0.5 μg); (3) ³²P-labelled 21 000-mol.wt. protein (1.5 μg) (4) unlabelled small and large basic protein (0.75 μg); (5) ³²P-labelled 17 000-mol.wt. protein (1.5 μg); (6) ³²P-labelled large basic protein (0.5 μg).

Table 2. *Time course of incorporation of ^{32}P into basic proteins of rat brain myelin*

Rats were anaesthetized with diethyl ether, injected with 1 mCi of $\text{H}_3^{32}\text{PO}_4$, and killed after 2, 4 or 24 h. Fractions enriched in basic proteins and purified basic proteins were prepared as described in the text. The specific radioactivity of myelin proteins was corrected to the value of ^{32}P on the first day of the experiment. Results are means of two separate experiments. Numbers of animals are given in parentheses. The abbreviations are defined in the legend to Table 1.

	Specific radioactivity (c.p.m./mg of protein)		
	2 h (10)	4 h (16)	24 h (10)
Fraction enriched in basic proteins	363 393	297 729	293 919
Fraction enriched in basic proteins after solubilization in SDS, conjugation with MDPF and dialysis against 0.1% SDS	201 777	153 991	184 998
21K	196 766	121 587	110 107
LPB	119 895	111 752	67 852
17K	193 041	204 669	177 462
SBP	220 440	210 857	172 517

Discussion

Phosphorylation of two basic proteins of rat brain myelin *in vitro* and *in vivo* was first demonstrated by Steck & Appel (1974) and Miyamoto & Kakiuchi (1974). Recently, Sulakhe *et al.* (1980*a,b*) showed phosphorylation of only large and small basic protein *in vitro* by protein kinase in myelin and myelin subfractions of rat brain. The present study has provided evidence that a host of proteins of rat brain myelin are labelled 2 h after intracerebral injection of $\text{H}_3^{32}\text{PO}_4$. Among those labelled were proteins migrating to the position of myelin-associated glycoprotein, Wolfgram protein(s), proteolipid protein and DM-20 (Agrawal *et al.*, 1972). Since these proteins were not purified and shown to contain covalently linked phosphoserine and phosphothreonine it remains to be established if they are phosphorylated. However, in SDS/polyacrylamide-gel systems the free phosphate and/or loosely bound phospholipid associated with the myelin proteins is likely to be dissociated and would therefore migrate with the dye front.

Since the four basic proteins of rat brain myelin were phosphorylated both *in vivo* and *in vitro* (M. M. Garwood, S. Becker, M. Landt & H. C. Agrawal, unpublished work), it is reasonable to suggest that the same protein kinase is probably responsible for their phosphorylation in both conditions. In addition, this is the first reported evidence of phosphorylation *in vivo* of four basic proteins of rat brain myelin. The high-molecular-weight 'polymer' in the isolated preparation of rat brain myelin basic proteins is particularly interesting (Fig. 6, arrows), because the 'polymer' has previously been shown to have the amino acid composition of, and induces experimental allergic encephalomyelitis like, myelin basic protein (Martenson & Deibler, 1975). More

recently, Golds & Braun (1978) also observed the generation of the high-molecular-weight 'dimer' from purified large basic protein. Since this 'polymer' was generated when purified small basic protein was eluted from the gel and subjected to re-electrophoresis (Fig. 6), the possibility of contamination of an unrelated high-molecular-weight protein co-migrating with small basic protein is ruled out. Identical results were obtained when 21 000-mol.wt. protein, large basic protein and 17 000-mol.wt. protein were repurified and fluorographed (results not shown). The relationship of the polymerization of basic proteins during purification and electrophoresis to the interaction of basic protein(s) *in vivo* is obscure at present.

The presence of labelled lipids in the basic protein-enriched fraction is supported by the following observations. First, when both undialysed and dialysed basic protein-enriched fractions were examined by SDS/polyacrylamide-slab-gel electrophoresis and the gel was fluorographed, a substantial amount of radioactivity was seen in the region where lipids are expected to migrate in the undialysed fraction. Second, there was no change in the electrophoretic pattern of proteins before and after dialysis, suggesting that the loss of radioactivity after dialysis is due to lipids that were co-extracted with myelin basic proteins when delipidated myelin was extracted with 0.1 M-HCl. Third, a substantial amount of radioactivity seen on top of acid-urea gels (Fig. 3*b*) was due to lipids that were dissociated from basic proteins by 8 M-urea. This radioactive material was found to migrate to the position of lipids when proteins in acid-urea disc gels was subsequently separated in the second dimension by SDS/polyacrylamide-slab-gel electrophoresis and the gel was fluorographed (results not shown). Thus it can be concluded that a substantial

amount of lipid is associated with myelin basic proteins. The dissociation of lipids from protein occurs only when basic protein is treated with strong denaturing agents like SDS and urea. The affinity of basic proteins for lipids, particularly acidic lipids *in situ*, may be of importance in stabilizing the structure of this unique multilamellar membrane.

Since the specific radioactivity of all four basic proteins of rat brain myelin is essentially similar (Table 2), the basic proteins are perhaps equally accessible to protein kinase *in vivo*. This observation suggests that the four basic proteins are present at the same locale within the multilamellar myelin membrane.

This work was supported in part by grants from NSF BNS-7824739 and NIH NS 13464. The authors are grateful to the Kroc Foundation for the gift of the Amino acid analyser, Deborah Leeke for amino acid analysis and Linda Mabry for typing the manuscript.

References

- Agrawal, H. C., Burton, R. M., Fishman, M. A., Mitchell, R. F. & Prensky, A. L. (1972) *J. Neurochem.* **19**, 2083–2089
- Agrawal, H. C., Trotter, J. L., Burton, R. M. & Mitchell, R. F. (1974) *Biochem. J.* **140**, 99–109
- Agrawal, H. C., Hartman, B. K., Shearer, W. T., Kalmbach, S. & Margolis, F. (1977) *J. Neurochem.* **28**, 495–508
- Allerton, S. E. & Pearlman, G. E. (1965) *J. Biol. Chem.* **240**, 3892–3898
- Allison, J. H., Agrawal, H. C. & Moore, B. W. (1974) *Anal. Biochem.* **59**, 592–601
- Barbarese, E., Carson, J. H. & Braun, P. E. (1978) *J. Neurochem.* **31**, 779–782
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Carnegie, P. R., Kemp, B. E., Dunkley, P. R. & Murray, A. W. (1973) *Biochem. J.* **135**, 569–572
- Carnegie, P. R., Dunkley, P. R., Kemp, B. E. & Murray, A. W. (1974) *Nature (London)* **249**, 147–150
- Dunkley, P. R. & Carnegie, P. R. (1974) *Biochem. J.* **141**, 243–255
- Fishman, M. A., Hagen, S., Trotter, J. L., O'Connell, K. & Agrawal, H. C. (1979) *J. Neurochem.* **32**, 1077–1083
- Golds, E. E. & Braun, P. E. (1978) *J. Biol. Chem.* **253**, 8171–8179
- Gonzales-Sastre, F. (1970) *J. Neurochem.* **17**, 1049–1056
- Hartman, B. K., Agrawal, H. C., Kalmbach, S. & Shearer, W. T. (1979) *J. Comp. Neurol.* **188**, 273–290
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 165–175
- Martenson, R. E. & Deibler, G. E. (1975) *J. Neurochem.* **24**, 79–88
- Martenson, R. E., Deibler, G. E. & Kies, M. W. (1969) *J. Biol. Chem.* **244**, 4268–4272
- Martenson, R. E., Deibler, G. E. & Kies, M. W. (1971) *J. Neurochem.* **18**, 2417–2426
- Martenson, R. E., Deibler, G. E., Kies, M. W., McKneally, S. S., Shapira, R. & Kibler, R. F. (1972) *Biochim. Biophys. Acta* **263**, 193–203
- Matthieu, J.-M. & Kuffer, A. D. (1978) in *Myelination and Demyelination* (Palo, J., ed.), pp. 159–170, Plenum Press, New York and London
- McNamara, J. O. & Appel, S. H. (1977) *J. Neurochem.* **29**, 27–35
- Mehl, E. & Halaris, A. (1970) *J. Neurochem.* **17**, 659–668
- Miyamoto, E. (1975) *J. Neurochem.* **24**, 503–512
- Miyamoto, E. (1976) *J. Neurochem.* **26**, 573–577
- Miyamoto, E. & Kakiuchi, S. (1974) *J. Biol. Chem.* **249**, 2769–2777
- Miyamoto, E. & Kakiuchi, S. (1975) *Biochim. Biophys. Acta* **384**, 458–465
- Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6335
- Nussbaum, J. L., Rouayrenic, J. F., Mandel, P., Jolles, J. & Jolles, P. (1974) *Biochem. Biophys. Res. Commun.* **57**, 1240–1247
- Petralli, E. H., Theissen, B. J. & Sulakhe, P. V. (1980) *Arch. Biochem. Biophys.* **105**, 520–535
- Randernath, K. (1970) *Anal. Biochem.* **34**, 188–205
- Steck, A. J. & Appel, S. H. (1974) *J. Biol. Chem.* **249**, 5416–5420
- Sulakhe, P. V., Petralli, E. H., Theissen, B. J. & Davis, E. R. (1980a) *Biochem. J.* **186**, 469–473
- Sulakhe, P. V., Petralli, E. H., Davis, E. V. & Theissen, B. J. (1980b) *Biochemistry* **19**, 5363–5371
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412