Enzymic Hydrolysis of Myelin Basic Protein and other Proteins in Central Nervous System and Lymphoid Tissues from Normal and Demyelinating Rats

By GEORGE F. BULETZA, JR. and MARION EDMONDS SMITH

*Department of Neurology, Veterans Administration Hospital, Palo Alto,
and Department of Neurology, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.*

(Received 11 December 1975)

Proteolytic activity of central-nervous-system tissue of the normal rat was examined over the pH range 2-9 with casein, haemoglobin and myelin basic protein as substrates. With casein as a substrate, brain and spinal cord homogenates showed very similar activity profiles with increasing pH, with the main peaks of proteolytic activity at pH 3-4 and 5-6. When haemoglobin was used, one broad main peak of activity from pH 3 to 5 was demonstrated. There was no optimum pH, however, for proteolytic activity with myelin basic protein as a substrate, and considerable hydrolysis was observed from pH 3.5 up to pH 8. Proteolytic activity at the various pH values was compared by using homogenates of spinal cords from rats with acute experimental allergic encephalomyelitis and those from rats injected with Freund's adjuvant alone. The profiles of activity were similar with peaks at pH 3.5 and 5.5 with casein as a substrate, but the specific activity was significantly higher at most pH values in the spinal-cord homogenates from rats with experimental allergic encephalomyelitis. Similarly the spinal-cord homogenates from these latter rats contained much more proteolytic activity toward myelin basic protein throughout the pH range than was present in the control spinal cords. Homogenates from lymph nodes of rats with experimental allergic encephalomyelitis and from those of the controls contained two to three times as much proteolytic activity as that of the central-nervous-system tissue and had a different proteolytic activity profile from that of the central nervous system, with higher activity at the neutral than at acid pH. The results are discussed with regard to the probability that inflammatory cells such as lymphocytes may be the cause of the increased proteolytic activity in the central nervous system of animals with experimental allergic encephalomyelitis, and that enzymes from these cells possess the capability of digesting myelin basic protein.

Proteolysis in brain was shown to be active with such substrates as casein and haemoglobin (Marks & Lajtha, 1971), and this activity can vary with pH according to the particular substrate used (Oja & Oja, 1970). Little is known, however, of the separate enzymes involved or of their endogenous substrates in the central nervous system. Einstein *et al.* (1968) have shown that basic protein from central-nervous-system myelin is a substrate for brain acid proteinase, but this type of study has not been extended to other enzymes.

Evidence suggests that increased proteolytic activities which may be associated with certain demyelination processes may be brought into the central nervous system by inflammatory cells (Smith *et al.*, 1974); therefore it was decided to examine the spectrum of proteolytic activity of spinal cord and lymphoid tissues of normal rats and of rats with experimental allergic encephalomyelitis by using several substrates, including purified myelin basic protein. A comparative study of enzymes of neural and

lymphoid origin and their role in acute inflammatory demyelination may contribute to the understanding of the basic mechanism of myelin dissolution.

Methods

Animals

Normal, untreated male rats were taken from the laboratory Wistar-rat colony maintained on Wayne Lab Chow and tap water *ad libitum*. For the experiments with rats with experimental allergic encephalomyelitis, Lewis rats of about 120 g, obtained from an animal supply house, were injected intradermally in both hind feet with a total of 0.1 mg of basic protein incorporated in 0.5 ml of Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) with 3 mg of *Mycobacterium tuberculosis* H37Ra (Difco)/ml of adjuvant; 100% incidence of acute experimental allergic encephalomyelitis was obtained in this manner.

Control rats were injected with Freund's adjuvant containing the added *Mycobacterium* alone.

Basic protein

The basic protein injected into rats was obtained from purified myelin prepared from guinea-pig spinal cord by the method of Smith (1968) but by using only a single density-gradient separation. The well-washed freeze-dried myelin was extracted for 30 min with 0.01 M-HCl in a motor-driven Teflon/glass homogenizer. The mixture was centrifuged at 17000g for 10 min, and the supernatant fluid was freeze-dried and used as the purified myelin basic protein for induction of experimental allergic encephalomyelitis in Lewis rats.

Because myelin basic protein prepared by the above method contained some lipid, the basic protein used as substrate was prepared somewhat differently. Purified myelin from Wistar-rat brain and spinal cord was prepared by the same method as described above. The washed, frozen myelin was homogenized in chloroform/methanol (2:1, v/v) in sufficient quantity to make a single phase, then filtered through a funnel containing glass wool in the stem. The filtrate was treated with 0.2 vol. of 0.88% (w/v) KCl, mixed well, centrifuged for 10 min at 1500g, and the aqueous phase removed by vacuum suction. The interface protein was removed with a glass rod and kept. Then a one-sixth vol. (upper phase) of 0.1 M-potassium citrate (Webster & Folch-Pi, 1961) was added, mixed, centrifuged for 10 min at 1500g, and again the aqueous phase was removed and the interface protein kept. The pooled interface protein was extracted twice by homogenizing for 30 min with 0.005 M-HCl, and the extracts were freeze-dried. Chromatography of the basic protein on silicic acid-coated glass plates by using chloroform/methanol/NH₃ (120:70:9, by vol.) as the liquid phase showed no material migrating from the protein that was stainable with iodine fumes; thus this preparation was presumed to be lipid-free.

Tissue preparation

The rats were decapitated, and the brains and spinal cords removed, immediately weighed and homogenized for 5 min in 20 vol. of 0.01 M-sodium phosphate buffer, pH 6.5 (Bowen & Davison, 1973), containing 0.1% Triton X-100, by using a motor-driven Teflon-glass homogenizer in a 4°C cold-room. The use of Triton X-100 was found to result in somewhat higher enzyme activity than in its absence. Spinal cords and inguinal and popliteal lymph nodes were removed from rats with acute experimental allergic encephalomyelitis and from control rats treated with Freund's adjuvant and homogenized as above.

Proteolytic-enzyme assays

Buffer solutions were 0.1 M in sodium acetate (pH 2.0–6.5) and sodium phosphate (pH 5.0–8.0). Sodium citrate (pH 2–5) and Tris/HCl (pH 7–9) were also compared, but the use of acetate and phosphate buffers resulted in the highest activity. Substrates used for proteinase assay were α -casein, 20 mg/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.); rat myelin basic protein, 20 mg/ml, homogenized in 0.001 M-HCl until clear; and urea denatured haemoglobin, 20 mg/ml (Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Preparation of the haemoglobin substrate was as follows: the haemoglobin was dissolved in 0.075 M-HCl at 80°C. The solution was homogenized with a motor-driven Teflon/glass homogenizer and incubated for 60 min at 37°C. The incubation mixtures consisted of 0.2 ml of protein substrate and 1.6 ml of buffer containing 0.1% Triton X-100. The ionic strength of the buffers prevented any change greater than 0.1 pH unit after the addition of tissue homogenates and substrates and subsequent incubation. The mixtures were contained in stoppered tubes and incubated at 37°C with shaking at 80 oscillations/min for 3 h. Control tubes included tissue blanks without protein substrates incubated for 3 h and a complete mixture with 1.0 ml of 20% (w/v) trichloroacetic acid added at zero time. The reaction was terminated after 3 h with 1.0 ml of 20% trichloroacetic acid. The precipitated material was removed by centrifugation, and the supernatant fluid filtered through glass wool and filter paper. Portions of the filtrate were assayed for ninhydrin-positive material (Moore & Stein, 1954) and for phenolic substances by the Folin-Ciocalteu procedure (Spies, 1957). Standard curves were prepared with tyrosine and the results expressed as μ mol of tyrosine per mg of protein. The protein content of samples of the homogenate was measured by the method of Lowry *et al.* (1951) with a human serum albumin standard, Versatol (General Diagnostics, Morris Plains, NJ, U.S.A.). The zero-time control values were subtracted from both values for the tissue blank and the experimental mixture. The difference between the test sample and the resulting tissue-blank control was taken as a measure of the enzyme activity. Assays were run on normal and Freund's adjuvant-treated controls and on rats with acute experimental allergic encephalomyelitis. Statistical evaluation of the significances of differences between group means used the Student-Fisher *t* test. The standard deviation from the mean was calculated as an expression of variance.

Results

Normal proteinase activities

The pH-dependence of the proteolytic activity of rat spinal-cord homogenate with casein, haemoglobin,

Fig. 1. Enzymic hydrolysis of casein (a), haemoglobin (b), and myelin basic protein (c) by spinal-cord homogenates from normal Wistar rats

Incubations were made at 37°C with 0.1 M-sodium acetate (●) and -sodium phosphate (▲) buffers. Amino acids released were measured by both the ninhydrin (—) and Folin-Ciocalteu (---) procedures. Activities of zero-time controls and tissue blanks were subtracted from total test activities, and results expressed as μmol of tyrosine equivalents per mg of protein $\pm$ S.E.M. (a), Six animals were used, and results are average values of duplicate sets of incubations; (b) and (c), four were used, and results are animals averages of duplicate incubations.

and basic protein as substrates is depicted in Figs. 1(a), 1(b) and 1(c). Comparison of proteolytic activity by assay of amino acids and peptides released into the supernatant solution by both the ninhydrin and the phenol methods revealed rather similar profiles, but the ninhydrin method resulted in much higher peaks. When casein was used as the substrate, two peaks of activity were obvious in both methods, one at pH 3.5, the other between pH 5 and 6, whereas the activity at pH ranges above 7 was very low. In agreement with the findings of Serra *et al.* (1972), the profile of protein hydrolysis by spinal-cord homogenates was remarkably similar to that of brain homogenates when measured by either the ninhydrin or the phenol method.

Proteolysis of haemoglobin by spinal-cord homogenates occurred mostly at acid pH, with a broad peak of activity between pH 2.5 and 6 and a secondary peak at pH 6.5, when assayed by the ninhydrin method (Fig. 1b). In contrast with the low pH required for haemoglobin proteolysis, hydrolysis of purified myelin basic protein by spinal-cord homogenate was active throughout the pH range studied, with only slightly higher activities in the acidic pH range (Fig. 1c).

Fig. 2. Comparison of the enzymic hydrolysis of casein by spinal-cord homogenates from Lewis rats with acute experimental allergic encephalomyelitis (○) and from Lewis rats treated with Freund's complete adjuvant (●)

Incubations were performed in 0.1 M-sodium acetate and sodium phosphate buffers at 37°C. Amino acid and peptides released were measured by the ninhydrin method. Four sets of animals were used, and results are averages of duplicate incubations (the vertical bars denoting the S.E.M.).

Fig. 3. Comparison of enzymic hydrolysis of myelin basic protein by spinal-cord homogenates from Lewis rats with acute experimental allergic encephalomyelitis (○) and from Lewis rats treated with Freund's complete adjuvant (●).

Incubations and measurements were performed as described in Fig. 2. Seven sets of animals were used; and results are averages of duplicate incubations (the vertical bars denoting the S.E.M.).

Proteinase activities in acute experimental allergic encephalomyelitis

In spinal cord of Lewis rats with acute experimental allergic encephalomyelitis, a moderate increase in proteinase activities over that of the Freund's-adjuvant-treated controls was measured at all pH values. Casein hydrolysis by spinal cords from rats with experimental allergic encephalomyelitis and by their Freund's-adjuvant-treated controls occurred with peaks of activity at pH 3.5 and 5.5, and resembled the normal pattern shown in Fig. 1a. At these activity peaks, casein hydrolysis by the tissue from rats with experimental allergic encephalomyelitis increased by 65% at pH 5.5 and by 20% at pH 3.5 (Fig. 2). In the less-active neutral and alkaline pH ranges casein hydrolysis by the spinal cord from rats with experimental allergic encephalomyelitis increased by 150–200% over that of the Freund's-adjuvant-treated controls. The differences between the proteolytic activity of the spinal cords from rats with experimental allergic encephalomyelitis and that from the controls were statistically significant

Fig. 4. Comparison of enzymic hydrolysis of myelin basic protein by pooled inguinal and popliteal lymph-node homogenates from Lewis rats with acute experimental allergic encephalomyelitis (○) and from Lewis rats treated with Freund's complete adjuvant (●).

Incubations and measurements were performed as in described Fig. 2. Four sets of animals were used, and results were averages of duplicate incubations (the vertical bars denoting the S.E.M.).

Fig. 5. Comparison of the enzymic hydrolysis of endogenous tissue proteins (tissue blanks) in homogenates of spinal cord (○) and of pooled inguinal and popliteal lymph nodes (●) from Lewis rats treated with Freund's adjuvant

Incubations and measurements were performed as described in Fig. 2. Vertical bars denote the S.E.M.

($P = <0.05$ or less) at all pH values between 3.5 and 8.0. It must be emphasized, however, that the increases found at neutral pH are due mostly to the low activities in normal and Freund's-adjuvant-treated control spinal cords in this pH region.

Basic-protein hydrolysis by homogenates of spinal cord of Lewis rats with acute experimental allergic encephalomyelitis was active throughout the pH range, at somewhat higher values than for the Freund's-adjuvant-treated controls (Fig. 3). The increase in proteolytic activity amounted to 63% at pH 4.5, with lesser increases throughout the entire range, but at all pH values through the range 2–7.5, the increases were statistically significant ($P = <0.05$ or less).

The hydrolysis of myelin basic protein in lymph-node homogenates from Lewis rats with acute experimental allergic encephalomyelitis was active, but showed little difference from that of lymph nodes of Freund's-adjuvant-treated controls (Fig. 4). The specific activities of the immunologically activated lymph nodes did not differ from those of normal lymph nodes (not shown), but the former lymph nodes weighed at least ten times as much as the normal. Proteolytic-activity peaks for lymph-node homogenates were at pH 4.0 and 7.0. The greater rate of basic protein hydrolysis by lymph tissue than by nerve tissue was noteworthy, especially in the neutral-pH area. Basic-protein hydrolysis in lymph-node homogenates at pH 7.0 was 230–255% higher than the activity found in spinal-cord homogenates (cf. Figs. 1c and 4), and similarly, the hydrolysis of endogenous proteins of lymph node was greatest at neutral pH (Fig. 5). Increases in the hydrolysis of endogenous

proteins by tissue homogenates from animals with acute experimental allergic encephalomyelitis were barely perceptible, and were not significantly different from the tissues from Freund's-adjuvant-injected animals for either spinal cord or lymph node.

Discussion

Normal proteolytic-enzyme activities in the central-nervous-system tissue were dependent on the pH, the substrate and the particular buffer used, as well as the method of assay. In the present study, several buffers and three substrates were compared, and two methods of assay were explored.

Proteolytic activity in spinal cord and in brain was highest in the acidic-pH ranges when casein and haemoglobin were substrates as previously reported for whole-brain homogenates by Oja & Oja (1970). Generally the present results agree with the proteolytic-activity profile shown by these workers using these substrates. In contrast with the low proteolytic activities seen at neutral pH with casein and haemoglobin, the hydrolysis of purified myelin basic protein by central-nervous-system tissues proceeded nearly as well at neutral as at acid pH, and in lymph-node tissue, basic protein was hydrolysed best at neutral pH, as was the endogenous protein in spinal-cord and lymph-node tissue.

Catabolic enzymes, proteolytic enzymes in particular, have long been regarded as a probable factor in myelin disruption. This idea has been strengthened by the observed breakdown of myelin basic protein by purified brain acid proteinase to form toxic, encephalitogenic peptides, and by reported increases of proteinase activities in various demyelinating conditions (Einstein, 1972). Acid proteinase has been identified as showing an increase at the edge of the multiple-sclerosis plaque with a concomitant loss of basic protein (Hallpike & Adams, 1969; Hallpike *et al.*, 1970; Einstein *et al.*, 1969, 1970; Riekkinen *et al.*, 1970). Increased activities of acid proteinase (Rauch *et al.*, 1973; Govindarajan *et al.*, 1974b; Hirsch & Parks, 1975), of cathepsin A and neutral proteinase (Smith *et al.*, 1974), and of cathepsin B₁ (Govindarajan *et al.*, 1974b) have been demonstrated in central-nervous-system lesions of monkeys and rats with experimental allergic encephalomyelitis.

Basic protein *in situ* in purified myelin is easily hydrolysed by acetylated trypsin (Wood *et al.*, 1974; Banik & Davison, 1974), and purified basic protein is hydrolysed by purified brain acid proteinase (Einstein *et al.*, 1968), Pronase, and elastase (M. E. Smith, unpublished work). When associated with myelin lipid, basic protein may be partially protected from the activity of proteolytic enzymes (London & Vossenberg, 1973).

Although central-nervous-system tissue contains an abundance of proteolytic enzymes, it is more likely

that the source for the increase of enzymes in experimental allergic encephalomyelitis or other inflammatory demyelinating conditions is the invading cells such as macrophages and lymphocytes (Stefanovic *et al.*, 1962; Lapresle, 1971; Clausen & Jorgensen, 1972; Rauch *et al.*, 1973; Smith *et al.*, 1974; Govindarajan *et al.*, 1974a). This hypothesis is supported by electron-microscopic evidence for the invasion of mononuclear cells between lamellae of the myelin sheath during demyelination in guinea pigs in experimental allergic encephalomyelitis (Lampert & Carpenter, 1965), and by electrophoretic and enzymic measurements indicating that lysosomal acid proteinase activity is both rich and localized in lymphocytes and macrophages (Stefanovic *et al.*, 1962; Lapresle, 1971). Hirsch & Parks (1975) have correlated increases in lysosomal enzymes, including acid proteinase and cathepsin A, with increased DNA concentrations, a measure of cellular invasion. Removal of the regional lymph node as long as 9 days after sensitization can prevent development of experimental allergic encephalomyelitis in the rabbit (Condie *et al.*, 1970).

These results confirm high proteolytic activity in lymphoid tissues and further indicate especially high activity at neutral pH. Although lymph-node homogenates hydrolyse basic protein, the activity was not shown to be altered with the development of experimental allergic encephalomyelitis when compared with homogenates from Freund's-adjuvant-treated controls or with homogenates of normal lymph nodes. Govindarajan *et al.* (1974a) found no difference in cathepsin B₁ and D activities between lymphocytes obtained from multiple-sclerosis patients and controls, and Smith *et al.* (1974) found no differences in cathepsin A and D activities between lymph nodes obtained from Lewis rats with experimental allergic encephalomyelitis and those from the controls. The high activity of proteolytic enzyme is, therefore, not specific for the demyelinating condition, and the specificity undoubtedly lies in the target of the invading cells. If myelin-sensitized cells from lymph node or their progeny migrate into the nervous tissue, they may be involved in the demyelinating process by becoming 'myelin aggressors', causing oligodendrocyte lysis and separation of myelin lamellae as demonstrated by Lumsden (1971) for myelin-sensitized circulating lymphocytes in multiple-sclerosis patients. If proteolytic enzymes were released, one might expect a greater release of neutral proteinase activity at the lesion site, since this is the proteolytic enzyme of greatest activity in the lymphoid tissue. Detection of this activity may be difficult because of the lability and the presence of inhibitors of neutral proteinases in the central nervous system (Vogel *et al.*, 1968; Buletza & Quay, 1971).

The present results demonstrate proteolytic activity in lymph nodes capable of hydrolysing myelin

basic protein, especially at physiological pH. This activity, however, does not appear to be specific to encephalitogen-challenged lymphocytes, but may be an innate property of lymph node-cells, which multiply greatly when activated.

This study was supported by a grant (no. NS-02785) from the National Institutes of Health, and by the Veterans Administration.

References

- Banik, N. L. & Davison, A. N. (1974) *Biochem. J.* **143**, 39–45
- Bowen, D. M. & Davison, A. N. (1973) *Biochem. J.* **131**, 417–419
- Buletza, G. F., Jr. & Quay, W. B. (1971) *Enzyme* **12**, 311–321
- Clausen, J. & Jorgensen, P. (1972) *Int. Arch. Allergy* **43**, 457–468
- Condie, R. M., Kelley, J. T., Thomas, L. & Good, R. A. (1957) *Anat. Rec.* **127**, 405
- Einstein, E. R. (1972) in *Handbook of Neurochemistry* (Lajtha, A., ed.), vol. 2, pp. 107–129, Plenum Press, New York
- Einstein, E. R., Csejtey, J. & Marks, N. (1968) *FEBS Lett.* **1**, 191–195
- Einstein, E. R., Csejtey, J., Davis, W. J., Lajtha, A. & Marks, N. (1969) *Int. Arch. Allergy* **36**, 363–375
- Einstein, E. R., Dalal, K. B. & Csejtey, J. (1970) *J. Neurol. Sci.* **11**, 109–121
- Govindarajan, K. R., Offner, H., Clausen, J., Fog, T. & Hyllested, K. (1974a) *J. Neurol. Sci.* **23**, 81–87
- Govindarajan, K. R., Rauch, H. C., Clausen, J. & Einstein, E. R. (1974b) *J. Neurol. Sci.* **23**, 295–306
- Hallpike, J. F. & Adams, C. W. M. (1969) *Histochem. J.* **1**, 559–578
- Hallpike, J. F., Adams, C. W. M. & Bayliss, O. B. (1970) *Histochem. J.* **2**, 199–208
- Hirsch, H. E. & Parks, M. E. (1975) *J. Neurochem.* **24**, 853–858
- Lampert, P. W. & Carpenter, S. (1965) *J. Neuropathol. Exp. Neurol.* **24**, 11–24
- Lapresle, C. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 135–155, North-Holland, Amsterdam
- London, Y. & Vossenberg, F. G. A. (1973) *Biochim. Biophys. Acta* **307**, 478–490
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lumsden, C. E. (1971) *Brain Res.* **28**, 365–390
- Marks, N. & Lajtha, A. (1971) in *Handbook of Neurochemistry* (Lajtha, A., ed.), vol. 5A, pp. 49–139, Plenum Press, New York
- Moore, S. & Stein, W. H. (1954) *J. Biol. Chem.* **211**, 907–913
- Oja, S. S. & Oja, H. (1970) *J. Neurochem.* **17**, 901–912
- Rauch, H. C., Einstein, E. R. & Csejtey, J. (1973) *Neurobiology* **3**, 195–205
- Riekkinen, P. J., Clausen, J., Frey, H. J., Fog, T. & Rinne, U. K. (1970) *Acta Neurol. Scand.* **46**, 349
- Serra, S., Grynbaum, A., Lajtha, A. & Marks, N. (1972) *Brain Res.* **44**, 579–592

- Smith, M. E. (1968) *Biochim. Biophys. Acta* **164**, 285–293
- Smith, M. E., Sedgewick, L. M. & Tagg, J. S. (1974) *J. Neurochem.* **23**, 965–971
- Spies, J. R. (1957) *Methods Enzymol.* **3**, 467–468
- Stefanovic, J., Webb, T. & Lapresle, C. (1962) *Ann. Inst. Pasteur* **103**, 276–284
- Vogel, R., Trautschold, I. & Werle, E. (1968) *Natural Proteinase Inhibitors*, p. 159, Academic Press, New York
- Webster, G. R. & Folch-Pi, J. (1961) *Biochim. Biophys. Acta* **49**, 399–401
- Wood, J. G., Dawson, R. M. C. & Hauser, H. (1974) *J. Neurochem.* **22**, 637–643