The Isolation and Properties of Experimental Allergic Encephalitogenic Protein

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1. Experimental allergic encephalitogenic (EAE) protein was isolated from ox spinal cord by a modification of the method of Martenson & LeBaron (1966). 2. The protein was examined by acrylamide-gel electrophoresis and its amino acid composition determined. 3. Sedimentation-velocity runs in the ultracentrifuge indicate a molecular weight of about 15 100 at pH 7.8, and calculation suggests that approx. 137 amino acid residues are present per molecule. 4. Gel-filtration and diffusion studies suggest that the protein is non-globular. 5. Optical-rotatory-dispersion measurements show the protein to have no helical secondary structure even at pH values near to the isoelectric point. In the presence of triphosphoinositide, changes in the optical rotatory dispersion of the protein could be interpreted to mean that it develops a small degree of secondary structure. 6. On treatment with cyanogen bromide the experimental allergic encephalitogenic protein is split into at least two fragments, the larger of which is only about 12% smaller than the parent protein and is antigenically active, and the smaller of which is devoid of antigenic activity.

The aim of the work presented in this paper was to prepare a sample of the basic protein of myelin, EAE protein,† which produces allergic encephalomyelitis when injected into experimental animals, so that this could be treated in model systems with the acidic phospholipids that also occur in myelin. Recently a number of methods have been reported for obtaining preparations of this protein, and it is obvious from the different properties reported by various groups of workers that the products are quite variable in size and composition. To some extent the situation has been clarified by the work of Nakao, Davis & Roboz-Einstein (1966*a,b*), which gave evidence that the EAE protein can be broken down enzymically during the isolation into smaller fragments, some of which retain their antigenic activity.

On examining some of the methods published for preparing EAE protein, we found that the procedure described by Martenson & LeBaron (1966) is very convenient for obtaining a good yield of protein that is relatively homogeneous and probably representative of the protein that actually exists *in vivo*. Their procedure was modified to obtain a somewhat

purer product, and this was examined by electrophoresis, ultracentrifugation and optical rotatory dispersion. Because of the low methionine content found on amino acid analysis of the preparation, the opportunity was taken to cleave it into smaller fragments by reaction with cyanogen bromide.

EXPERIMENTAL

Preparation of EAE protein. Protein having encephalitogenic activity was prepared by a modification of the procedure of Martenson & LeBaron (1966). Fresh ox spinal cords were obtained from a local slaughterhouse within a few hours of the death of the animals and chilled on ice for transport to the laboratory. After the removal of the meninges and adhering blood vessels the cords were either worked up immediately or stored frozen for periods up to 4 weeks before use. All operations were carried out at 4°. The spinal-cord tissue (100g.) was homogenized in 11. of chloroform-methanol (2:1, v/v) to remove lipids, and the residue was collected by centrifugation. The residue was washed with three 300ml. lots of chloroform-methanol (2:1, v/v), and then treated with 300ml. of acetone and recovered by filtration under reduced pressure. The dried residue was extracted twice by stirring it for 20 min. with 300 ml. of water and recovered each time by centrifuging.

The residue was suspended in 50 ml. of ice-cold 0.03 N-HCl for 1 hr. and the suspension centrifuged at 10 000*g* for 15 min.; this extraction was repeated. The combined acid extracts were adjusted to pH 7–7.5 with ice-cold 0.5 N-NaOH.

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† Abbreviation: EAE protein, experimental allergic encephalitogenic protein.

Insoluble material was removed by centrifuging for 15 min. at 10000g.

A column of 3g. of DEAE-cellulose (2100; Macherey, Nagel and Co., Düren, Germany), which had been washed with water until the pH of the suspension was 6.5–7, was prepared. The fraction soluble at pH 7 obtained from the acid extract was passed through the DEAE-cellulose column, the column was washed with water, and the unadsorbed material was dialysed overnight against water at 4°. The dialysed extract was freeze-dried and dissolved in 3–5 ml. of 0.1 M-NaCl, and any insoluble material was removed by centrifuging. Samples were clear, although they often had a slightly yellow-brown colour, and were applied to a 1.5 cm. × 100 cm. column of Sephadex G-150 that had been well washed with 0.1 M-NaCl. The column was eluted with 0.1 M-NaCl, with or without added 0.05 M-tris-HCl buffer, pH 7.5. Fractions (3 ml.) were collected and protein was detected by measurement of its extinction at 280 or 230 m μ . The centre portion of the major peak having an elution volume approximately twice the void volume of the column was collected and stored frozen, with or without the removal of salts by dialysis. The yield of purified EAE protein was 200–300 mg./100 g. of fresh tissue.

The procedure of Kibler, Fox & Shapira (1964) was also used for the preparation of EAE protein. The acid extract of acetone-defatted ox spinal cord was fractionated on a column of CM-cellulose (2100; Macherey, Nagel and Co.) by elution with a gradient of NaCl (0–0.5 M) at pH 8 as described by Kibler *et al.* (1964). The bulk of encephalitic material was found in one peak eluted with approx. 0.20–0.25 M-NaCl, giving the preparation as described by Kibler *et al.* (1964). This was then further purified by gel filtration on Sephadex G-75: the material from 100 g. of tissue was passed through a column (1.5 cm. × 75 cm.) and eluted with 0.1 M-NaCl–0.05 M-tris-HCl, pH 7.5. Minor peaks were discarded and the major peak was saved. This procedure yielded approx. 12–20 mg. of protein/100 g. of fresh tissue; however, the behaviour of the extract on CM-cellulose and the quantity of the final product were quite variable.

Amino acid analysis. Determination of the amino acid composition of EAE protein hydrolysates was carried out by ion-exchange chromatography on a column (0.6 cm. × 100 cm.) of sulphonated polystyrene with gradient elution over 18 hr. in a Technicon automatic analyser. Samples containing 5–8 mg. of protein were hydrolysed in 90% (v/v) formic acid–12 N-HCl (1:1, v/v) under reflux. Corrections for hydrolytic losses and slow release were made by hydrolysing samples for 24, 48 and 72 hr. and extrapolating to zero time. Tryptophan was measured from the molar ratio of tyrosine to tryptophan determined as described by Beaven & Holiday (1952). Amide nitrogen was not determined.

Acrylamide-gel electrophoresis. The procedure followed was essentially that of Carnegie, Lamoureux & Bencina (1967b). Gels (15%, w/v) were prepared in a Perspex trough (18 cm. × 10 cm. × 0.6 cm.). The mixture contained 18 g. of Cyanogum 41 (British Drug Houses Ltd., Poole, Dorset) and 1.2 ml. of 10% (v/v) *NNN'*-tetramethylethylenediamine made up to a total volume of 120 ml. with degassed water. Ammonium persulphate (1.2 ml. of a 2%, w/v, solution) was added, and the solution was quickly poured into the trough which was covered (care being taken to exclude all air) with a lid in which were set six teeth

(9 mm. × 4 mm. × 1 mm.) located 5 cm. from one end to act as slot moulds. After setting for 15–20 min. the gel was placed in 500 ml. of acetic acid–formic acid–water (4:1:45, by vol.) containing 4 M-urea for at least 24 hr. before use. Electrophoresis was carried out at 4° in a Shandon universal tank, with the same buffer, pH 2.5, as above but without urea. Sheets of synthetic cellulose sponge (approx. 4 mm. thick) were used as connecting wicks. Samples (0.04 ml. of a solution of 0.3–1.5 mg. of protein/ml. of water) were placed in the slots and the electrophoresis was started at 35–40 ma and 125–140 v. After 30–45 min. the gel was covered with a thin polyethylene film, and electrophoresis was continued for 12–16 hr.

Gels were stained for 4 hr. with acetic acid–methanol–water (1:5:5, by vol.) containing 0.05% of Amido Black 10B and 0.5% of HgCl₂. Destaining was achieved by prolonged washing with the same solvent. The final treatment consisted of prolonged soaking in 1% (v/v) glycerol, and the gels were stored wrapped in thin polyethylene film.

The proportion of protein in each band of a sample was estimated by the procedure of Johns (1967). The stained protein bands were cut from the gel, weighed, placed in stoppered tubes and broken up into fine particles with a glass rod. Blanks in the appropriate weight range were cut from a clear portion of the same gel. Dimethyl sulphoxide (3 ml.) was added to each tube and they were incubated overnight at room temperature. After centrifuging, the colour in the clear supernatants was measured in a spectrophotometer at 600 m μ . The colour yields of the various bands were expressed as percentages of the total colour yield for the sample, assuming a similar concentration-dependent stain absorption for all bands.

Molecular-weight determinations. The molecular weights of EAE protein preparations were compared by gel filtration on a column (1.5 cm. × 100 cm.) of Sephadex G-75 (Andrews, 1964). The column was eluted with 0.1 M-NH₄Cl, pH 2.0, as described by Lowden, Moscarello & Morecki (1966), and calibrated with globular proteins of known molecular weight: serum albumin, chymotrypsinogen A, soya-bean trypsin inhibitor, cytochrome c, insulin and glucagon.

The molecular weight of EAE protein prepared by the modification of the method of Martenson & LeBaron (1966) was also determined from sedimentation and diffusion characteristics in a Beckman model E analytical ultracentrifuge with schlieren optics. A synthetic-boundary cell was used for all runs, this being of a special capillary type for the determinations at high pH. Both the sedimentation coefficient and the diffusion coefficient were determined for several preparations of EAE protein at several protein concentrations. The EAE protein was dialysed for 36 hr. against three changes of water at 4°, and then for 24 hr. against the buffer used.

Optical rotatory dispersion. EAE protein was examined in solutions of various ionic strengths and pH values in a Polarmatic 62 (Billingham and Stanley Ltd., London). The protein at a concentration of 2.2 mg./ml. was examined alone or in the presence of 1.52 μ moles of triphosphoinositide/ml. over a spectral range of 294–590 m μ with a 10 cm.-light-path cell. Triphosphoinositide alone gave no rotation between 294 and 590 m μ . The results were analysed both by the method of Moffitt & Yang (1956) and by that of Shechter & Blout (1964).

Cyanogen bromide cleavage. The procedure followed was

that of Press, Piggot & Porter (1966). The protein sample was taken up in 25 vol. of 70% (v/v) formic acid; CNBr (4mg./mg. of protein) was added and the mixture was incubated at room temperature for 24–36 hr. The reaction mixture was diluted with 10 vol. of water and freeze-dried. The CNBr collected in the acetone–solid CO₂ trap was decomposed with NaOH.

The reaction products were separated on a 1.5 cm. × 100 cm. column of Sephadex G-75 eluted with 0.1 M-acetic acid–sodium acetate buffer, pH 5.

Protein analysis. Protein in aqueous solution was measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951) with a serum albumin standard.

Antigenic activity of EAE protein. For the determination of experimental allergic encephalitogenic activity, the test animals were male guinea pigs (300–400 g.). The inoculum consisted of 1 part (by vol.) of antigen in 0.1 M-NaCl solution and 1 part of Freund's adjuvant containing 2 mg. of *Mycobacterium butyricum*/ml. (Difco Laboratories, Detroit, Mich., U.S.A.). The inoculum was heated to 60° for 10 min. and homogenized by repeated rapid ejection from a syringe. The mixture (0.1 ml.) was injected intradermally over the sternum. Animals were observed daily for onset of clinical symptoms, which included weight loss, ataxic gait, hind-limb paralysis, faecal and urinary incontinence, tremors and death. Symptoms generally appeared between 10 and 18 days. Surviving animals were killed at 30 days. Information available from this limited bioassay procedure was qualitative only, and served principally to indicate the presence or absence of antigenic activity in a preparation or fraction.

RESULTS

Behaviour of EAE proteins on Sephadex gel. The fraction soluble at pH 7, described by Martenson & LeBaron (1966), gave several peaks when subjected to gel filtration on Sephadex G-150. It was observed that treatment of the crude fraction with DEAE-cellulose at neutral pH, as described by Kies (1965), followed by dialysis decreased but did not eliminate both the high- and the low-molecular-weight contaminants. Passage through Sephadex G-150 was then used as a final purification step. The elution patterns on Sephadex G-75 columns of the EAE protein fractions prepared by the method of Kies (1965) and by the present modification of the method of Martenson & LeBaron (1966) were very similar (Fig. 1). When compared with a series of globular standard proteins by the method of Andrews (1964) they indicated an apparent 'molecular weight' of 40 000–50 000, though the asymmetric peak of the preparation by the method of Kies (1965) suggested the presence of some lower-molecular-weight material in it. In contrast, the elution volume of the preparation by the method of Kibler *et al.* (1964) was greater and indicated a 'molecular weight' of approximately 10 000–12 000 (Fig. 1).

Kies, Alvord, Martenson & LeBaron (1966) reported a 'molecular weight' of 35 000–40 000 by gel filtration on Bio-Gel P-10 of EAE protein

Fig. 1. Elution patterns of various EAE protein preparations on a Sephadex G-75 column. The column (1.5 cm. × 100 cm.) was eluted with 0.1 M-NH₄Cl, pH 2. EAE protein was prepared (A) by the method of Kies (1965), (B) by the present modification of the Martenson & LeBaron (1966) procedure, and (C) by the method of Kibler *et al.* (1964).

prepared essentially by the procedure of Martenson & LeBaron (1966), and similarly Nakao *et al.* (1966a,b) reported a 'molecular weight' of 37 000 for their own preparation, using elution with 0.1 M- or 1.0 M-acetic acid from Bio-Gel P-10. Lowden *et al.* (1966) found an apparent molecular weight of 10 000 by elution at pH 2.0 for an EAE protein isolated from purified myelin, and Carnegie, Bencina & Lamoureux (1967a) reported an apparent molecular weight of 15 000 for the protein by elution at pH 2 from Sephadex G-50.

The above results indicated a similarity of behaviour on gel columns between our preparation and the 'high-molecular weight encephalitogens' of other workers. In view of the difficulties in obtaining a true molecular weight for non-globular proteins by this method, more extensive investigations were not made.

Acrylamide-gel electrophoresis. Fig. 2 shows the electrophoretic patterns on acrylamide gel at pH 2.5 produced by samples of EAE protein, prepared by three different methods and all active in producing the disease (Table 1). A sample of cytochrome c (Sigma Chemical Co., St Louis, Mo., U.S.A.) was run for comparison and the mobilities of the other peaks were compared with this standard, in accord with the suggestions of Carnegie *et al.* (1967b). An estimate of the staining intensity of the bands was determined by the method of Johns (1967). The sample prepared by the modification of the procedure of Kies (1965) showed two major bands at M_{cyt} (mobility relative to cytochrome c) 0.64 (65%) and 0.87 (30%) as well as a number of minor bands (Fig. 2b, sample 3). Two major bands were also observed on starch-gel electrophoresis of

Fig. 2. Electrophoresis of various EAE protein preparations on 15% acrylamide gel at pH 2.5. (a) (2) 28 μ g. of cytochrome *c*; (1), (3) and (4) EAE protein prepared by the present modification of the Martenson & LeBaron (1966) procedure: (1) 28 μ g.; (3) 14 μ g.; (4) 7 μ g. (b) (1) 25 μ g. of cytochrome *c*; (2) 5 μ g. of EAE protein prepared by the method of Kibler *et al.* (1964); (3) 63 μ g. of EAE protein prepared by the method of Kies (1965); (4) 50 μ g. of EAE protein prepared by the present modification of the method of Martenson & LeBaron (1966).

Table 1. *Encephalitogenic activity of EAE protein preparations*

Activities refer to materials prepared in this investigation by the methods of the given references. For the method of assay of activity, see the text.

Tissue from which EAE protein was prepared and method	Dose of EAE protein (μ g.)	Incidence of clinical disease
Guinea-pig brain (Kies, 1965)		
Major peak of Sephadex G-150	5	7/8
Faster-running component from preparative starch-gel electrophoresis at pH 5	10	4/5
Slower-running component at pH 5	10	5/5
Ox brain white matter (Kibler <i>et al.</i> 1964)	25	8/10
Ox spinal cord (Kibler <i>et al.</i> 1964)	25	4/4
Ox spinal cord (Martenson & LeBaron, 1966)	50	5/5
	25	5/5
	10	5/5
	5	4/5
EAE protein treated with CNBr		
Control sample	25	4/7
Large component	25	4/7
Small component	25	0/7

this preparation, and, when these were partially separated by a preparative technique (Murray, 1962), both fractions were active experimental allergic encephalitogenic antigens when tested in guinea pigs (Table 1). The sample prepared by our modification of the method of Martenson & LeBaron (1966) showed a major band at M_{cyt} 0.64 (at least 80%) which corresponded to the slower running of the bands of the EAE protein prepared by the Kies (1965) method, a secondary band at M_{cyt} 0.77 (less than 20%) and some very minor faster-running components (Figs. 2a and 2b). Although Martenson & LeBaron (1966) reported that their preparation

was electrophoretically homogeneous in acrylamide gel, our own observations and also the more recent experience of R. E. Martenson (personal communication) suggest that their preparation was indeed heterogeneous. Although the EAE protein prepared by the method of Kibler *et al.* (1964) with further purification (see the Experimental section) gave a single fast-running band at M_{cyt} 1.10 the amount of material used in the run was limited (5 μ g.) and the presence of minor components might not have been detected by the staining procedure.

Because of the very poor and variable yield of

low-molecular-weight protein given by the Kibler *et al.* (1964) method and the greater heterogeneity of that from the Kies (1965) method, subsequent work was done on the EAE protein prepared by the present modification of the Martenson & Lebaron (1966) procedure. The electrophoretic purity of this last-named preparation is very similar to that described by Roboz Einstein & Csejtey (1967) for EAE protein prepared by the method of Nakao *et al.* (1966a,b).

Ultracentrifugation studies. Both the sedimentation coefficient and the diffusion coefficient were determined for several preparations of EAE protein at several protein concentrations. The determinations were made in phosphate-sodium chloride buffer, pH 7.8 and I 0.1, and both sedimentation and diffusion coefficients were extrapolated to infinite dilution ($S_{20,w}^0$ 1.33s; $D_{20,w}^0$ 7.80×10^{-7} cm.²/sec.). In the ultracentrifuge, the protein gave a single symmetrical peak, indicating that the minor components observed on acrylamide-gel electrophoresis must be similar in size to the main component of the preparation. This agrees with the homogeneous behaviour of the preparation on gel filtration with Sephadex G-75 (Fig. 1).

The specific volume was estimated to be 0.72 ml./g. from the amino acid composition (Table 2) and the specific volumes of amino acid residues given by Cohn & Edsall (1943). From this value of the specific volume and the above sedimentation and diffusion coefficients, a molecular weight of 15100 was calculated. This is a somewhat larger value than the 12500 obtained by Nakao *et al.* (1966a,b) for their fraction G in the ultracentrifuge at pH 4.6 and the even smaller value reported by Carnegie *et al.* (1967a) at pH 5.5. The frictional ratio calculated from the sedimentation and diffusion coefficients (1.7) suggests that the EAE molecule is asymmetric.

The determination of the molecular weight of highly charged proteins is a complex matter (e.g. see Walker, 1965). The EAE protein was observed to be isoelectric and to be precipitated readily at about pH 10.5. Attempts were made to determine the molecular weight at pH 11.0 in 0.05 M-glycine-sodium hydroxide buffer containing 0.25 M-sodium chloride (higher salt concentrations causing precipitation), to make measurements on a less charged molecule. The protein was not as insoluble at this pH as it was when its solution was adjusted to just below the isoelectric point. To avoid alkaline degradation of the protein (Lumsden, Robertson & Blight, 1966; Caspary & Field, 1965) the solution of protein at a concentration of less than 1% in water was dialysed against the pH 11.0 buffer just before the run was performed. The protein exhibited a single symmetrical peak, indicating that no breakdown was occurring under these conditions. The

Table 2. *Amino acid composition of EAE protein fraction*

Amino acid	Amino acid composition	
	Number of moles/100 moles of total residues	Number of residues/molecule of protein* (nearest integer)
Arginine	10.8	15
Histidine	5.9	8
Lysine	7.6	10
Total basic	24.3	33
Aspartic acid	7.1	10
Glutamic acid	6.4	9
Total acidic	13.5	19
Threonine	4.5	6
Serine	9.5	13
Proline	7.7	10-11
Glycine	13.6	18-19
Alanine	7.5	10
Valine	1.9	3
Methionine	1.3	2
Isoleucine	1.8	2-3
Leucine	6.1	8
Phenylalanine	5.0	7
Tyrosine	2.6	3-4
Tryptophan	0.7	1
Total residues	100.0	135-139
Ammonia	6.5	9

* Calculation based on an assumed molecular weight of approx. 15100.

results obtained at this higher pH indicated a change in the diffusion coefficient of the protein from the value obtained at pH 7.8 without a corresponding variation in the sedimentation coefficient. A possible explanation of this phenomenon is that some aggregation of the protein molecules was occurring; this would affect the diffusion coefficient, but the sedimentation coefficient would be unchanged since it would be derived from observations on the non-aggregated particles. This explanation was borne out by an equilibrium-sedimentation run at pH 11 at a protein concentration of 0.43%. The slope of the plot for determining z_{av} , molecular weight increased towards the bottom meniscus, suggesting some aggregation. The molecular weight determined from the top-meniscus region of the plot was 15340, which agrees well with the determination made at pH 7.8.

Amino acid composition. Table 2 shows the averaged amino acid composition of EAE protein preparations isolated by the modification of the method of Martenson & LeBaron (1966). The analysis is closely similar to those presented by Martenson & LeBaron (1966) (component soluble at

Table 3. *Parameters obtained from optical-rotatory-dispersion measurements on EAE protein*

pH	Concn. of NaCl (M)	Triphospho- inositide (μ mole/mg. of protein)	Moffitt & Yang (1956) treatment (λ_0 212 m μ)		Shechter & Blout (1964) treatment			
			$[\alpha]_{546}$	b_0	$A_{(\alpha,p)193}$	$A_{(\alpha,p)225}$	Helix content (%)	
							H_{193}	H_{225}
7.5	0.01	—	-141	0	-545	-228	6	15
7.5	0.50	—	-116	-10	-298	-292	12	18
8.0	0.05	—	-119	0	-342	-278	11	17
10.0	0.05	—	-116	0	-419	-203	9	13
12.0	0.05	—	-110	0	-369	-203	10	13
Unbuffered	0.025	—	-108	0	-336	-234	11	15
Unbuffered	0.025	0.7	-69	-67	+73	-334	23	20

pH 7), Nakao *et al.* (1966a,b) (fraction G) and also Carnegie *et al.* (1967a). It shows a high basic amino acid (24.3 moles/100 moles of residues) and glycine (13.6) content but no cysteine. The high glycine and low isoleucine content distinguish this basic protein from the usual histone type of protein found in animal tissues (Nakao *et al.* 1966a,b; Butler, Power & Palau, 1967). The molar percentage of basic amino acid present is considerably higher than that reported by Lowden *et al.* (1966) for the EAE protein that they isolated from ox brain myelin fractions.

Carnegie *et al.* (1967a) calculated the number of residues of each amino acid, presumably on the assumption of one tryptophan residue per molecule. This calculation suggested a protein of 149 residues with a molecular weight of 16500. A similar calculation based on the assumption of a molecular weight of approximately 15100 is given in Table 2 and suggests a protein of about 135–139 residues. It has to be emphasized that this is subject to error because the protein preparation is not completely homogeneous. Nevertheless, since the minor contaminant is also a basic protein of similar molecular weight, which may be derived from the main protein (see below), it is probable that the error introduced is not serious. The greatest difficulty is in trying to assign a reliable molecular weight on which to base the calculations.

Optical rotatory dispersion. Table 3 shows the parameters obtained by the mathematical treatment of the optical-rotatory-dispersion measurements by the methods of Moffitt & Yang (1956) and of Shechter & Blout (1964). The b_0 values in the Moffitt & Yang (1956) plots indicate that the EAE protein has a negligible helical content, and the low helical content suggested from the Shechter & Blout (1964) plots (H_{193} and H_{225}) confirm this conclusion. This applies to measurements made at pH values between 7.5 and 12 and sodium chloride concentrations up to 0.5 M.

The relationships between the $A_{(\alpha,p)193}$ and

$A_{(\alpha,p)225}$ values (given in Table 3) when compared with the plots given by Shechter & Blout (1964) suggest that no other type of secondary structure is present.

Both mathematical treatments suggest that, when sufficient triphosphoinositide is added to form a solubilized complex of EAE protein and the phospholipid (Palmer & Dawson, 1969), a small amount of secondary structure develops that is not shown by either the individual phospholipid or the protein (Table 3). However, it is not possible to know whether this small change in the optical rotatory dispersion can be interpreted as meaning that a small part of the peptide chain in the complex is present in the helical form.

A similar apparent increase in secondary structure was observed for highly basic proteins (histones) when solubilized in an excess of detergent (Jirgensons, 1966). Since triphosphoinositide has good detergent properties it is likely that the two observations are connected. Jirgensons (1966) attributed the effect on histones to the existence of a more compact arrangement of protein molecules inside the detergent-solubilized micelles, presumably as a result of neutralization of the electrical charges on the protein.

Splitting of EAE protein by cyanogen bromide. The low methionine content of EAE protein (two residues per molecule) suggested that it might be possible to break the protein into a limited number of smaller fragments by the use of cyanogen bromide, which specifically splits proteins at methionine residues (Gross & Witkop, 1961). When the method of Press *et al.* (1966) for cyanogen bromide cleavage was applied to the present EAE protein preparation, two fragments were obtained on gel filtration of the product on Sephadex G-75 and examination of the fractions by extinction at 280 m μ (Fig. 3) or by the Lowry *et al.* (1951) protein determination. The major fragment (87% by u.v. absorption of the protein recovered from the column) was retarded slightly on the Sephadex

Fig. 3. Elution pattern of EAE protein on Sephadex G-75 after cleavage with CNBr. A column (1.5 cm. $\times$ 100 cm.) was eluted with 0.1 M-sodium acetate-acetic acid buffer, pH 5. ○, EAE protein (50 mg.) before treatment (E_{280}); ●, EAE protein (90 mg.) after reaction with CNBr (E_{280}); Δ, EAE protein (90 mg.) after reaction with CNBr (protein determined by the method of Lowry *et al.* 1951).

Fig. 4. Electrophoresis on 15% acrylamide gel of EAE protein after cleavage with CNBr. (1) High-molecular-weight fragment (20 μ g.) isolated by gel filtration from CNBr-treated EAE protein. (2) EAE protein (20 μ g.) before treatment.

column compared with the unchanged protein. This would suggest that it is only marginally smaller than the original protein. Studies in the analytical

ultracentrifuge at pH 7.8 indicated a single symmetrical peak; however, the molecular weight derived from sedimentation and diffusion results was approximately the same as that of the starting material. Acrylamide-gel electrophoresis indicated that, like the starting material, the large fragment contained a contaminating protein of slightly greater electrophoretic mobility (Fig. 4). The major component of the fragment itself exhibited a mobility only slightly greater than the major component of the starting material, in fact the same as the mobility of the contaminant in the starting material.

The other product of cyanogen bromide cleavage was eluted at the end of the gel column, suggesting that it represents one or several small polypeptides. Amino acid analysis of the smaller fragment indicated 13 amino acids, which suggests a decrease in the molecular weight of the starting EAE protein of only 11%. Encephalitogenic activity was found in the large fragment only and not in the smaller polypeptide fragment (Table 1). Obviously the methionine residues must be near the end(s) of the molecule and the antigenicity is not grossly affected by cleavage at this position(s). Cyanogen bromide cleavage was therefore not a very useful tool for locating the antigenic site.

DISCUSSION

It is apparent, from the differing results obtained by many investigators on the properties of EAE protein present in brain and spinal cord, that a number of different protein structures can act as satisfactory antigens for producing the disease in experimental animals (e.g. see Nakao *et al.* 1966a,b; Lumsden *et al.* 1966). A good deal of the confusion in the field has been resolved by the demonstration by Nakao *et al.* (1966a,b) that proteolytic enzymes present in brain tissue can hydrolyse the main encephalitogenic protein present *in vivo* into smaller fractions during the isolation and that some of these also show the same antigenic activity. This breakdown appears to be largely prevented when chloroform-methanol is used to extract the tissue initially, as in the Martenson & LeBaron (1966) procedure, whereas defatting with acetone allows some proteolysis to occur unless the extracting medium is adjusted to below pH 2.0, the method used by Nakao *et al.* (1966a,b).

The EAE protein isolated by the present modification of the method of Martenson & LeBaron (1966) therefore probably represents the protein as it exists *in situ* in the myelin sheath. Its high basic amino acid content and behaviour on gel electrophoresis indicate a molecule that would have a large net positive charge at physiological pH values,

and from precipitation studies the isoelectric point appears to be about pH 10.5. The ultracentrifugal results indicate a molecular weight of about 15100 in aqueous media at pH 7.8. The number of amino acid residues calculated from this molecular weight and the amino acid analysis (Table 2) is about 137. Both Nakao *et al.* (1966*a,b*) and Carnegie *et al.* (1967*a*) reported slightly higher molecular weights (approx. 16000) based on amino acid analysis and tryptic digestion of their protein preparations. Both groups, however, reported much lower molecular weights from ultracentrifugal data obtained at an acid pH. The behaviour of the protein in the diffusion studies suggests that it is not globular, since values for the diffusion coefficient would be more in keeping with a globular protein with a molecular weight of about 30000. This conclusion is also supported by the relatively high frictional ratio. From the optical-rotatory-dispersion studies it is apparent that the protein has virtually no helical or other type of secondary structure, even at high pH values when the protein has minimal positive charge or in the presence of salt. Thus in solution the free protein probably exists as a non-globular polypeptide with a predominantly random-chain conformation. This would help to explain the behaviour of the EAE protein on gel filtration at pH 2, which indicates an apparent molecular weight of about 40000–50000. The elution of proteins from Sephadex columns can be correlated better with the Stokes ionic radius than with the molecular weight (Siegel & Monty, 1966). A non-globular hydrated protein tends to have a larger effective molecular radius and would be expected to exhibit a higher apparent molecular weight on gel columns that had been calibrated with globular proteins.

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