Isolation and partial characterization of 'trace' proteins and immunoglobulin G from cerebrospinal fluid

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Human cerebrospinal fluid investigated by immunoelectrophoresis with an antiserum against human cerebrospinal fluid has been shown to contain two proteins, which cannot be demonstrated in human serum (Clausen, 1961; MacPherson and Cosgrove, 1961; Hochwald and Thorbecke, 1962). These proteins will here be named β-trace protein and γ-trace protein according to Hochwald and Thorbecke (1962). Other investigators have found, however, from none to nine different precipitation arcs present in cerebrospinal fluid but not in serum when using the same technique (Dencker and Swahn, 1962; Dencker, 1963; Laterre and Heremans, 1963; Laterre, Heremans, and Carbonara, 1964). Because of the different results reported in the literature as regards the immunoelectrophoretic demonstration of proteins in human cerebrospinal fluid not observable in serum, the real existence of such proteins has to be doubted until they have been isolated and characterized in other ways than with immunoelectrophoresis only.

Tourtelotte, Parker, and Haerer (1964), as well as Caspary (1965), have used anion exchange chromatography in order to isolate immunoglobulin G (7S γ-globulin, IgG) from cerebrospinal fluid. However, these authors have not tested the fractions obtained for the presence of β-trace protein and γ-trace protein. As will be shown here the IgG fraction is always contaminated with the trace proteins after isolation by ion exchange chromatography on D.E.A.E.-cellulose.

The present work confirms the finding of β-trace protein and γ-trace protein in human cerebrospinal fluid. In addition an isolation procedure for these proteins and for IgG from cerebrospinal fluid is described.

MATERIAL AND METHODS

COLLECTION OF CEREBROSPINAL FLUID Cerebrospinal fluid for immunization was obtained from 'normal' patients, e.g., patients who visited the neurological department or were admitted to a psychiatric department because of headache, uncharacteristic vertigo, and slight psychoneurotic disturbances. Anamnestic data for other diseases were missing, and a thorough somatic and neurological investigation performed before lumbar puncture was normal in all patients. Lumbar puncture was carried out with a technique described by Antoni (1923) according to the modification of Höök (1957). Between 10 and 20 ml. cerebrospinal fluid was taken from each patient. Cell counting was performed within 30 minutes after puncture. The samples were centrifuged at 3,000 r.p.m. for 20 minutes at room temperature. The total protein content was determined according to Lowry's method (Daughaday, Lowry, Rosebrough, and Fields, 1952), as modified by Lous, Plum, and Schou (1956). Samples containing more than 3 red blood cells and/or 3 mononuclear cells per mm³, and with a total protein content exceeding 45 mg./100 ml., were excluded. The cerebrospinal fluid was concentrated immediately after centrifugation by means of ultrafiltration in collodion tubes at +7°C to a final concentration of 2.5% (w/v) and was then stored at −20°C.

Cerebrospinal fluid for immunization was also obtained from patients with multiple sclerosis diagnosed according to the criteria given by Müller (1949). These specimens were handled in the same way as 'normal' cerebrospinal fluid. In addition paper electrophoresis was performed on each sample. Before immunization the samples were combined in three pools: multiple sclerosis cerebrospinal fluid pool I with a γ-globulin content below 15%; pool II with a γ-globulin content between 15 and 25%; pool III with a γ-globulin content above 25%. Pool I consisted of cerebrospinal fluid from 20 patients, pool II from 22 patients, and pool III from eight patients. Serum was taken from each of these patients, stored at −20°C, and combined before immunization in three pools, each consisting of samples from the same patients as the three different pools of multiple sclerosis cerebrospinal fluid.

Cerebrospinal fluid for protein isolation was obtained by lumbar or cisternal puncture on patients hospitalized for different neurological diseases. All cerebrospinal fluid available containing fewer than 10 red blood cells per mm³ but otherwise independent of protein content or number of mononuclear cells was used for the experiments. The cerebrospinal fluid was concentrated to 3.5% (w/v) and stored as a pool at −20°C.

1 Apparatus obtained from Membranfiltergesellschaft, Göttingen, West Germany.
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PREPARATION OF ANTISERA  Four rabbits, each weighing about 3 kg., were immunized with concentrated ‘normal’ cerebrospinal fluid. One rabbit was immunized with multiple sclerosis-cerebrospinal fluid pool I, one with pool II, and one with pool III. In addition, one rabbit was immunized with each of the three different serum pools. All immunizations were performed in the same way, that is, subcutaneously with 0-75 ml. concentrated cerebrospinal fluid or 0-4 ml. serum mixed with Freund’s complete adjuvant in the proportions 3:1. The injections were repeated with the same doses every tenth day, and after at least six injections the rabbits were bled by heart puncture or by the ear and serum tested for the existence of antibody by means of immunoelectrophoresis. A satisfactory antibody content was considered to exist when the precipitation pattern obtained was comparable with that of a commercial antisera against human serum obtained from Behringwerke, Marburg/Lahn, West Germany. Additional immunization was carried out in case of need. Equal aliquots of sera from the four rabbits immunized with ‘normal’ cerebrospinal fluid were combined.

A specific antisera against β-trace protein and γ-trace protein was obtained through absorption of the prepared antisera against ‘normal’ cerebrospinal fluid with normal human serum according to a method described by Rosenthal and Soothill (1962). The different antisera against multiple sclerosis cerebrospinal fluid and multiple sclerosis serum were absorbed with normal human serum with the same technique.

Rabbit antisera against total human serum and specific antisera against human IgG, immunoglobulin M (γ1M-globulin, IgM), and immunoglobulin A (γ2A-globulin, IgA) were obtained from Behringwerke, Marburg/Lahn, West Germany.

PROTEIN SEPARATION PROCEDURES  Cross-linked dextran gel (Sephadex G-200, 40-120μ, or Sephadex G-75, 40-120μ, Pharmacia, Uppsala, Sweden) was treated by swelling for about 72 hours in excess of 0-1 M Tris-HCl buffer, pH 8.0 containing 1 M NaCl. After thorough equilibration with this buffer a column with the dimensions 1-0 × 110 cm. was packed according to the directions of the manufacturer. Before gel filtration the protein sample was dialysed with stirring in the cold room for 24 hours in 2 litres of 0-1 M Tris-HCl buffer, pH 8.0 containing 1 M NaCl. Buffer was changed once. Methanol was added to all solutions in the proportions of 1:10.00. The sample was applied as a distinct layer on top of the gel. The flow rate was 2-6 ml/hr/cm.² column cross section and was regulated with a pump or a Mariotte flask. Fractions were collected in a fraction collector at regular intervals of 30 or 60 minutes. The effluent was continuously monitored at 254 μm with a Uvicord (LKB-Produkter, Stockholm, Sweden), and/or absorbancy at 280 μm was measured for each tube. All protein determinations were made according to Lowry (Daughaday et al., 1952). Appropriate pooled fractions were concentrated to 3-5% (w/v) by means of ultrafiltration. The fractionation as well as subsequent concentration was performed in the cold room.

D.E.A.E.-cellulose¹ or D.E.A.E.-Sephadex² was washed by a cycle of dilute HCl and dilute NaOH and was then equilibrated for at least 24 hours with the buffer to be used on chromatography. A column with an internal diameter of 1 cm. was packed to a height of 10 to 15 cm. Columns with D.E.A.E.-cellulose were packed under slight air pressure. The samples containing cerebrospinal fluid proteins were dialysed against the buffer to be used and concentrated by ultrafiltration in the cold room to 3-5% (w/v) before application on the column. The chromatography was carried out in the cold room with the same equipment as on Sephadex. Elution of the column was made at a rate of 10 to 20 ml./hour.

ANALYTICAL METHODS  Immuno- and electrophoresis was carried out according to the method of Grabar and Williams, applying a modified microtechnique of Scheiddegger (1955). One per cent agar³ and a continuous buffer system of sodium veronal, pH 8.6 and ionic strength 0-05, was used. The wells were filled with 2 μl. of concentrated cerebrospinal fluid or with an equal amount of serum. Electrophoresis was run in a moist chamber for 75 minutes at approximately 9 V/cm. Subsequently, a central trough, 6.5 × 0.1 cm., was cut out at a distance of 3 mm. from the wells and filled with 30 μl. of antisera. Diffusion was allowed to proceed in a moist chamber for 20 hours. The slides were then washed in saline for one day and afterwards in distilled water for one hour. The slides were then covered with filter paper and dried at room temperature over night. Subsequently, the slides were stained with Amido black (Amido black 10 B, 10 g.; glacial acetic acid, 20 ml.; distilled water, 980 ml.) for 10 minutes, after which excess of dye was rinsed off in a bath of glacial acetic acid, methanol, and distilled water in the proportions of 10:45:45.

Agar gel electrophoresis was performed on microscopic slides according to Wieme (1959). A 0-9% agar solution and a continuous buffer system of sodium veronal, pH 8-4 and ionic strength 0.05, was used. A central trough was cut out and filled with 10 μl. of concentrated cerebrospinal fluid or 4 μl. of serum. Electrophoresis was run in a cooled chamber⁴ for 25 minutes at 200 volts (approximately 27 V/cm). Subsequently, the slides were fixed for at least 30 minutes in a solution containing ethanol, distilled water, and glacial acetic acid in the proportions of 70:25:5. The slides were then covered with filter paper and dried, whereupon they were stained with Amido black (Amido black 10 B, 0.5 g.; mercuric chloride, 5.0 g.; glacial acetic acid, 5 ml.; distilled water, 95 ml.). Excess of dye was rinsed off in a bath of glacial acetic acid and distilled water in the proportions of 1:20.

Starch gel electrophoresis was carried out according to Smithies (1959) but in horizontal trays and using 0-035 M glycine buffer, pH 8-8, containing 8 M urea in the gel as described by Cohen and Porter (1964). The final pH of the gel was 7-7-5. Electrode vessels contained 0.3 M

¹Brown Comp., Berlin, New Hampshire, U.S.A.
²A-50 medium, Pharmacia, Uppsala, Sweden.
³Difco Special Agar-Noble.
⁴Apparatus obtained from Vitatron N.V., Holland.
serum against 'normal' cerebrospinal fluid absorbed with normal human serum, the existence of the trace proteins in all samples of cerebrospinal fluid could be confirmed (Fig. 1C). The precipitation arc of \( \gamma \)-trace protein was weaker and more difficult to observe than the precipitate corresponding to \( \beta \)-trace protein.

On immunoelectrophoresis of cerebrospinal fluid with specific commercial antiserum against human serum IgG, IgM, or IgA no precipitates corresponding to the trace proteins could be demonstrated.

All samples of cerebrospinal fluid constituting multiple sclerosis-cerebrospinal fluid pool I, II, and III were investigated with the antisera obtained on immunization of rabbits with these pools. The \( \beta \)-trace protein and \( \gamma \)-trace protein could always be demonstrated. When immunoelectrophoresis was carried out with these antisera absorbed with normal human serum, no precipitation arc in addition to the trace proteins could be observed.

All sera from patients with multiple sclerosis used for immunization were also investigated by immunoelectrophoresis with the corresponding specific antiserum. No obvious discrepancies were noticed for immunization with 'normal' human sera.

**Results**

**Identification and Characterization of Trace Proteins in Cerebrospinal Fluid by Immuno-electrophoresis**

Immunoelectrophoresis was performed on cerebrospinal fluid from more than one hundred 'normal' patients and from 80 cases of multiple sclerosis, using antiserum against 'normal' cerebrospinal fluid. In these experiments two precipitation arcs could always be demonstrated, which obviously correspond to \( \beta \)-trace protein and \( \gamma \)-trace protein (Fig. 1A). Serum from the patients was always examined at the same time and in no case could the trace proteins be observed. On immunoelectrophoresis where commercial antiserum against human serum was used, these two proteins were never identified (Fig. 1B). On immunoelectrophoresis with antiserum against 'normal' cerebrospinal fluid absorbed with normal human serum, the existence of the trace proteins could be confirmed (Fig. 1C). The precipitation arc of \( \gamma \)-trace protein was weaker and more difficult to observe than the precipitate corresponding to \( \beta \)-trace protein.

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![Graph](image)

**Fig. 2.** Fractionation of 1-4 ml. concentrated human cerebrospinal fluid (3.5% w/v upper curve) and of 0.8 ml. human serum (lower curve) on the same 110 × 0.9 cm. column of Sephadex G-200 in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 M NaCl. The elution flow rate was 6 ml./hour and 2 ml. fractions of the effluent were collected.

when compared with the immunoelectrophoretic pattern obtained with commercial antiserum against human serum.

**Isolation and Electrophoretic Characterization of Trace Proteins from Cerebrospinal Fluid.** An elution curve with four peaks was obtained by gel filtration of 1-4 ml. concentrated cerebrospinal fluid on Sephadex G-200. Under identical conditions human serum gave an elution curve with three peaks (Fig. 2). Immunoelectrophoresis with antiserum against 'normal' cerebrospinal fluid showed that fractions corresponding to the second half of peak III from the cerebrospinal fluid curve contained β-trace protein in addition to albumin. The middle portion of peak IV usually contained only β-trace protein and γ-trace protein, while fractions from the last third of peak IV consisted of γ-trace protein. The amount of pure γ-trace protein isolated in an experiment as carried out according to Fig. 2 was about 0.3 mg. of 49 mg. protein applied.

Fractions from the second half of peak III and some of the following fractions of the serum elution curve (Fig. 2, lower curve) were concentrated together to 3.5% (w/v) and investigated on immunoelectrophoresis with the antiserum against 'normal' cerebrospinal fluid. No precipitation arcs corresponding to β-trace protein or γ-trace protein could be demonstrated.

Agar gel electrophoresis on fractions correspond-

![Agar gel electrophoresis](image)

**Fig. 3.** Agar gel electrophoresis of protein fractions obtained by gel filtration of human cerebrospinal fluid on Sephadex G-200 (compare Fig. 2, upper curve). A Fractions from peak II. B Fractions from peak III. C Fractions from the last third of peak IV.

ning to the middle portion of peak IV of the cerebrospinal fluid elution curve showed two bands in the β- and γ-region (Fig. 3). These bands are identical with the trace proteins as shown on immunoelectrophoresis. On starch gel electrophoresis in 8 M urea two distinct bands cathodic to the application line could be demonstrated when concentrated fractions from peak IV were run (Fig. 4). These bands are also identical with the trace proteins, as β-trace protein as well as γ-trace protein could be demonstrated in these fractions on immunoelectrophoresis when performed with antiserum against 'normal' cerebrospinal fluid, but not with antiserum against human serum. Part C in Fig. 4 corresponds to the last third of peak IV and shows practically pure γ-trace protein but no β-trace protein cathodic to the application line. In all electrophoretic runs shown in the figure there is, however, some contamination with albumin, which could not be excluded completely in this gel filtration experiment and could also be demonstrated on immunoelectrophoresis.

In order to purify the trace proteins further and to establish a reproducible method for their quantitation all fractions containing β-trace protein and γ-trace protein after gel filtration of cerebrospinal fluid on Sephadex G-200 were pooled, concentrated, and run on a Sephadex G-75 column. An elution curve with three peaks was obtained (Fig. 5). On immunoelectrophoresis performed with antiserum against 'normal' cerebrospinal fluid it was demon-
FIG. 4. Starch gel electrophoresis of protein fractions obtained from gel filtration of concentrated human cerebrospinal fluid on Sephadex G-200 (compare Fig. 2, upper curve). A Middle part of peak III. B. Middle part of peak IV. C Last third of peak IV.

FIG. 5. Optical density at 280 mμ of the effluent from gel filtration on Sephadex G-75 of 5 mg. protein obtained from the combined fractions of the last third of peak III and of peak IV from gel filtration of 30 mg. cerebrospinal fluid proteins on Sephadex G-200 (compare Fig. 2, upper curve). Column dimensions were 113 × 1 cm. and the buffer used was 0·1 M Tris HCl, pH 8.0, containing 1 M NaCl. The elution flow rate was 6 ml./hour and 3 ml. fractions of the effluent were collected. Shaded areas indicate fractions that were separately combined, concentrated and studied with immunoelectrophoresis (compare Fig. 6).

FIG. 6. Immunoelectrophoresis of fractions obtained by gel filtration on Sephadex G-75 of cerebrospinal fluid proteins (compare Fig. 5). Antigens: A Fractions corresponding to peak I. B Fractions corresponding to peak II. C Fractions corresponding to peak III. Antiserum against 'normal' human cerebrospinal fluid.
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strated that fractions corresponding to peak I contained mainly albumin and no trace proteins, while peaks II and III consisted of \( \beta \)-trace protein and \( \gamma \)-trace protein, respectively (Fig. 6). The gel filtration shown in Fig. 5 corresponds to a pool of 30 mg. cerebrospinal fluid protein that was run primarily on a Sephadex G-200 column. The fraction recovered from this run containing trace proteins and applied on the Sephadex G-75 column amounted to 5 mg. The yield of \( \beta \)-trace protein and \( \gamma \)-trace protein was 1-5 mg. and 0-2 mg. respectively. This means that the concentration of the two trace proteins in the referred pool of cerebrospinal fluid was about 5% and that \( \beta \)-trace protein dominated in amount over \( \gamma \)-trace protein.

**ISOLATION OF IgG FROM CEREBROSPINAL FLUID**

In order to isolate IgG from cerebrospinal fluid several experiments were carried out with chromatography of concentrated cerebrospinal fluid on D.E.A.E.-cellulose using phosphate buffers of different molarity (0-002-0-02) and pH (8-2-6-5). In all these experiments IgG was eluted in the first peak together with the trace proteins.

The fractions from the second peak obtained when human cerebrospinal fluid is gel filtrated on Sephadex G-200 (Fig. 2, upper curve) contained IgG but no trace proteins as revealed by immunoelectrophoresis. These fractions were combined and concentrated by ultrafiltration in the cold room to 3.5% (w/v). For the concentration procedure Erlenmeyer flasks were used containing 2 litres of the buffer to be used on chromatography, e.g., 0-01 M phosphate, pH 7-6, in order to dialyse the sample at the same time as concentration was done. The sample was then applied on a column with D.E.A.E.-cellulose or D.E.A.E.-Sephadex. The elution method finally adopted was with 0-01 M phosphate buffer, pH 7-6.

One distinct peak was obtained (Fig. 7) which on immunoelectrophoresis gave only one precipitation arc, identical with IgG when using antiserum against human serum, antiserum against 'normal' human cerebrospinal fluid, and antiserum against IgG (Fig. 8). The yield of pure IgG was about 40% of the original amount of \( \gamma \)-globulin applied on the Sepha-

**FIG. 7.** Chromatographic separation on D.E.A.E.-cellulose of 40 mg. pooled cerebrospinal fluid protein obtained from peak II on Sephadex G-200 (Compare Fig. 2, upper curve). Column dimensions were 15 x 1 cm. and the elution buffer was 0-01 M phosphate, pH 7-6. The elution flow rate was 20 ml./hour and 4 ml. fractions of the eluate were collected.

**FIG. 8.** Immunoelectrophoresis of (A) concentrated protein fractions corresponding to peak II obtained by gel filtration of cerebrospinal fluid on Sephadex G-200 (Compare Fig. 2, upper curve); (B-C) concentrated protein fractions corresponding to the first peak from a D.E.A.E.-cellulose chromatogram of the same material (compare Fig. 7). A-B Antiserum against 'normal' human cerebrospinal fluid. C. Antiserum against human serum IgG.
dex G-200 column as determined by paper electrophoresis.

DISCUSSION

When using an antiserum against 'normal' cerebrospinal fluid, β-trace protein and γ-trace protein could be identified on immunoelectrophoresis in all samples of cerebrospinal fluid investigated from 'normal' individuals and from patients with multiple sclerosis. Several individual samples of cerebrospinal fluid from patients with different neurological diseases as well as all pools of cerebrospinal fluid also always showed the presence of the two trace proteins when investigated with the same technique. These results are substantially in accordance with some earlier investigations (Clausen, 1961; MacPherson and Cosgrove, 1961; Hochwald and Thorbecke, 1962). Laterre and Heremans (1963) and Laterre et al. (1964), however, found nine precipitation arcs in cerebrospinal fluid when investigating different pathological cerebrospinal fluids on immunoelectrophoresis with their antisera against human cerebrospinal fluid absorbed with normal human serum. These authors found two arcs in the β- and γ-area dominating the immunoelectrophoretic pattern. These arcs may correspond to β-trace protein and γ-trace protein. The presence of the remaining seven precipitation arcs may be due to the existence in cerebrospinal fluid of proteins of very low concentration, being demonstrable only when a very potent antiserum against cerebrospinal fluid is used. Electrophoretic investigations on agar gel and starch gel (Figs. 3 and 4) of fractions from peak IV from a Sephadex G-200 run (Fig. 2, upper curve) as well as of fractions corresponding to peaks II and III from gel filtration on Sephadex G-75 (Fig. 5) show only two distinct bands, which are identical with β-trace protein and γ-trace protein. If the proteins demonstrated on immunoelectrophoresis by Laterre and Heremans have a molecular weight below that of albumin, they must exist in a very low concentration, as no additional bands can be seen in agar or starch gel electrophoresis in spite of the fact that the solutions applied on the gels had a protein concentration of 3-5%.

Immunoelectrophoretic investigations with antisera obtained through immunization of rabbits with cerebrospinal fluid from patients with multiple sclerosis have been described earlier (MacPherson and Cosgrove, 1961; Dencker, 1963). The results reported here agree mostly with those of MacPherson and Cosgrove (1961) and seem to indicate that cerebrospinal fluid in multiple sclerosis does not contain any 'specific' proteins in an amount sufficient to provoke the production in rabbits of antibodies, which can give visible precipitates on immunoelectrophoresis. The same can be argued as regards the possible existence of 'specific' proteins in serum from patients with multiple sclerosis.

Attempts have been made to isolate β-trace protein and γ-trace protein from cerebrospinal fluid by means of salt-fractionation procedures and anion exchange chromatography. The methods used led to considerable protein losses and the separated fractions were usually mixed with other proteins (MacPherson, 1962; Hochwald and Thorbecke, 1963). Gel filtration of concentrated human cerebrospinal fluid on columns containing Sephadex G-200 and Sephadex G-75 is a safe and reproducible method for the isolation and relative quantitation of β-trace protein and γ-trace protein. The method described is applicable to investigation of these proteins in samples of cerebrospinal fluid from individual patients. According to preliminary results it also seems possible to isolate the two proteins in one step by gel filtration on Sephadex G-75.

The gel filtration experiments demonstrate that the molecular size of both trace proteins is smaller than that of albumin, and that γ-trace protein has an apparent molecular size smaller than that of β-trace protein.

The existence of β-trace protein or γ-trace protein in human serum could not be demonstrated in the experiments described here. However, Hochwald and Thorbecke (1962) could identify γ-trace protein in concentrated human serum, and further investigation seems to be necessary before this question is definitely settled. Other problems which arise and demand investigation concern the probable relationship between the trace proteins and different subunits of IgG as well as urinary proteins of low molecular weight (for survey see Berggård, 1965).

Tourtellotte et al. (1964) and Caspary (1965) have tried to isolate IgG from cerebrospinal fluid by means of ion exchange chromatography on D.E.A.E.-cellulose. These authors have, however, not tested their fractionation products for the presence of β-trace protein and γ-trace protein. Our experiments with chromatography of concentrated cerebrospinal fluid on D.E.A.E.-cellulose using phosphate buffers of different molarity and pH have shown that it is not possible to obtain pure IgG in this manner because of contamination with the trace proteins. The two-step procedure described here, e.g., gel filtration on a column of Sephadex G-200 followed by chromatography of the IgG-containing fractions on a small column of D.E.A.E.-cellulose or D.E.A.E.-Sephadex, makes it possible to obtain pure IgG from cerebrospinal fluid in a yield of 40%. This yield is based on the value of γ-globulin as determined by paper electropho-
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It is assumed, however, that about one third of this γ-globulin value is assigned to the trace proteins. The yield of IgG from the two-step procedure should thus be calculated on a corresponding lower γ-globulin value and consequently be increased to about 60%.

**SUMMARY**

Two proteins of low molecular size have been isolated from human cerebrospinal fluid by gel filtration and have been characterized by means of immunoelectrophoresis, agar gel, and starch gel electrophoresis. In addition, immunoglobulin G from cerebrospinal fluid has been isolated in pure form by means of a two-step procedure involving gel filtration and anion exchange chromatography. The author is greatly indebted to Professor Ragnar Müller and Dr. John Sjöquist for advice and criticism. The investigation was supported by grants from the Swedish League Against Multiple Sclerosis, the Swedish Cancer Society, and the Swedish Medical Research Council (grant number W-261).

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