to the sera of groups 1, 2 and 3 to test for neutralization of antibody to BSA. Antibody to BSA was neutralized only in serum from mice injected with FTT plus anti-BSA (group 3) demonstrating that the observed anti-BSA in group 3 was of rabbit origin while the anti-BSA demonstrated in groups 1 and 2 was of mouse origin.

Summary. An enhanced antibody response to bovine serum albumin and fluid tetanus toxoid was found in mice when these antigens were injected in slight antigen excess with specific heterologous antisera. The specificity of the enhanced response was limited to specific antigens in complex. An enhanced response was also demonstrated to rabbit gamma globulin by testing the capacity of passively immunized mice to degrade I^{131}-labeled RGG at an accelerated rate.

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Use of an Antiserum Against Cerebrospinal Fluid in Demonstration of Trace Proteins in Biological Fluids.‡ (27113)

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The proteins of serum(1) and cerebrospinal fluid(2,3,4) have been extensively studied by immunoelectrophoresis with the aid of antisera prepared against human serum. Since protein concentration in cerebrospinal fluid (CSF) is much lower than in serum, it is necessary to concentrate CSF 50-to-100-fold before comparable immunoelectrophoresis diagrams can be obtained. Recently, antisera against such concentrated CSF have been prepared and examined by Chevance(5), Clausen(6) and in this laboratory. Such antisera reveal, upon immunoelectrophoresis of CSF, precipitation lines not seen in the diagrams obtained with human serum.

The present studies were undertaken in an attempt to determine whether the proteins responsible for these lines are 1) specific for CSF or 2) present in such small quantities in biological fluids that concentration of such fluids is necessary before these proteins can be demonstrated by immunoelectrophoresis. In contrast to the conclusion of Clausen(6), the results of immunoelectrophoretic and Ouchterlony plate studies performed with antisera to CSF after "absorption" with plasma or serum, using concentrated CSF, ascitic fluid, pleural fluid, urine, and plasma fractions obtained by starch block electrophoresis as the antigens, indicate that the proteins are not specific for CSF but can be found in small quantities in serum and other biological fluids.

Materials and methods. Preparation of antisera. Cerebrospinal fluid was obtained from patients admitted to the Neurological and Psychiatric Services of Bellevue Hospital, New York City. All grossly normal fluids were pooled and concentrated to a protein content of approximately 35 mg per ml by means of vacuum dialysis (Membranfiltergesellschaft, Göttingen, Germany). They were then mixed with equal parts of complete Freund's adjuvant and injected into the footpads of white New Zealand rabbits (2 ml of

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concentrated CSF per rabbit). This procedure was repeated once 3 weeks later. Four weeks later, a schedule of weekly bleedings and intravenous or intraperitoneal injections was started and continued for 2 months.

Antisera to human serum or plasma were prepared in a similar manner(7).

"Absorption" of anti-CSF antiserum with serum or plasma was carried out by adding 0.05 ml increments of plasma or of acute phase serum to 1 ml of anti-CSF. Precipitates were removed after incubation for 30 minutes at 37° and 2 hours at 4° C. This procedure was repeated 8 times. Absorption with CSF was performed similarly.

Starch block electrophoresis was performed on 20 ml of normal human plasma (400 V. for 24 hr) and on 4 ml concentrated CSF, original volume 300 ml, (300 V. for 40 hr) by the method of Kunkel and Slater(8). The proteins were eluted from blocks of starch cut at 1 cm for CSF and at 2.5 cm intervals for plasma. The γ- and β-globulin fractions from plasma were pooled separately and each reconcentrated by lyophilization to about 1 ml. The starch block fractions from CSF were reconcentrated in pools of 4 or 5 tubes.

Immunoelectrophoresis was performed according to the microtechnic described by Scheidegger(9). Special staining methods employed were performed according to Uriel (10). Routine protein staining was done with Amido Black 10B. Reactivity with Fe^{59} was tested(11). Double diffusion in agar was performed according to Ouchterlony (12).

Ascitic fluid was obtained from 2 patients, one with cirrhosis of the liver (concentrated 25-fold) and the other with an abdominal metastatic carcinoma (concentrated 6-fold). *Pleural fluid,* obtained from a patient with congestive heart failure was concentrated 10-fold. Proteins from 2000 ml *normal urine* were precipitated with 40% saturated $(NH_4)_2SO_4$ and redissolved in 2 ml of 0.15 M saline.† Another preparation of urinary proteins was obtained by vacuum dialysis (concentrated 1500 times).

Individual samples of CSF (concentrated 75-to-100-fold) were prepared from patients with normal spinal fluid and others with elevated spinal fluid proteins due to various neurological disorders, including meningovascular syphilis, multiple sclerosis, meningioma, glioma, metastatic carcinoma, encephalopathy of unknown etiology, multiple myeloma, and cerebral arteriosclerosis.

Results. Immunoelectrophoresis: Each of 20 individual normal and abnormal spinal fluids tested, concentrated either by lyophilization or by vacuum dialysis, showed 2 additional precipitation lines in the immunoelectrophoretic diagrams developed with antiserum to CSF and not seen with anti-human serum. These 2 lines were the only ones remaining after "absorption" of the anti-CSF with human plasma (0.4 ml plasma per 1 ml antiserum). One of the lines (β_{tr}-) extended from the slow albumin to the middle of the gamma globulin, and the other (γ_{tr}-) was in the gamma globulin region (Fig. 1A). γ_{tr}-protein showed a mobility similar to or somewhat slower than that of γ-globulin, varying with individual CSF samples. Some normal samples of concentrated CSF showed a double curvature of the γ_{tr}-line. "Absorption" of one of the antisera to CSF resulted in an antiserum which revealed only the γ_{tr}-protein in immunoelectrophoresis of CSF. Absorption of the anti-CSF with unconcentrated CSF (0.8 ml per ml of antiserum) diminished the capacity of the anti-CSF to form the β_{tr}- and γ_{tr}-lines in immunoelectrophoresis to a greater extent than absorption with twice as much serum. Further dilutions did not prove suitable for use in immunoelectrophoresis, and were, therefore, studied only in Ouchterlony plates (see below). Diluted and reconcentrated serum showed no lines in immunoelectrophoresis with absorbed anti-CSF.

It was next attempted to determine whether β_{tr}- and γ_{tr}-proteins are specific for CSF. The examination of immunoelectrophoretic diagrams prepared from ascitic fluid (concentrated 25-fold), and from normal urinary proteins (vacuum dialysis) with "absorbed" anti-CSF revealed the presence of both β_{tr}- and γ_{tr}-proteins in the ascitic fluid and in urine (Fig. 1B). Urinary proteins prepared by 40% saturated $(NH_4)_2SO_4$ pre-

† Kindly supplied by Dr. E. C. Franklin, Dept. of Medicine, New York Univ. School of Med.

cipitation contained only the β_{tr}- and not the γ_{tr}-protein. (Fig. 1F). These results suggested that traces of these proteins should also be found in plasma, although none of the 5 antisera prepared against human serum in this laboratory showed comparable precipitation lines with CSF in immunoelectrophoresis.

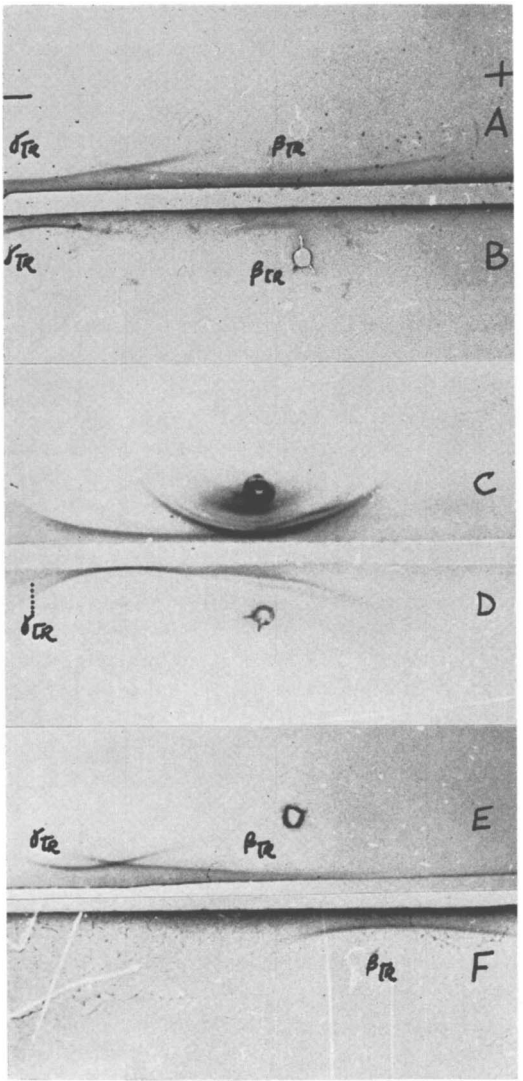


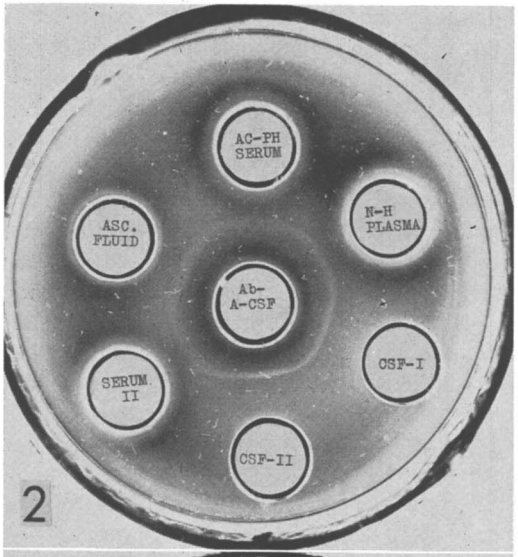
FIG. 1. Immunoelectrophoresis of various biological fluids developed with a 1:4 "absorbed" (A, B, E, F) or unabsorbed (C, D) antiserum to CSF. A, concentrated CSF (75×); B, concentrated ascitic fluid (20×); C, concentrated plasma from starch block fraction II; D, concentrated plasma from starch block fraction I; E, urinary proteins concentrated (1500×) by vacuum dialysis; F, concentrated urinary proteins (1000×) precipitated by $(NH_4)_2SO_4$. Both γ_{tr}- and β_{tr}-proteins are seen in CSF, ascitic fluid, and urine (concentrated by vacuum dialysis). In serum only γ_{tr}-protein could be visualized with this technic.

Plasma proteins cannot be concentrated effectively without previous fractionation. The β- and γ-globulin fractions obtained by starch block electrophoresis of plasma were, therefore, concentrated and examined by immunoelectrophoresis. The results with starch block electrophoresis of CSF showed that the corresponding fractions of CSF contained the β_{tr}- and γ_{tr}-proteins indicating similar electrophoretic mobilities on starch block and agar gel for these proteins. The immunoelectrophoretic diagrams prepared with anti-CSF from plasma fractions showed that γ_{tr}-protein was present in the γ-globulin fraction, but no evidence could be obtained for the presence of β_{tr}-protein in plasma (Fig. 1C, D).

Ouchterlony plates. When concentrated CSF (10×), pleural fluid (10×) ascitic fluid (6×), and β-globulin and γ-globulin starch block fractions of CSF were used as antigens with "absorbed" anti-CSF in the center well, formation of 2 precipitation lines occurred with each of the CSF fractions. The line closest to the antigen wells was more pronounced with the γ-globulin fraction, and the inner line more obvious with the β-globulin CSF fraction. The outer line showed immunological identity with the single line formed between the antiserum and CSF, ascitic fluid and the pleural fluid, indicating the presence of detectable amounts of γ_{tr}- in these fluids. The "absorbed" anti-CSF specific for γ_{tr}-protein developed only this outer line.

More concentrated ascitic fluid consistently showed 2 lines in the Ouchterlony plates with the antiserum to both β_{tr}- and γ_{tr}-proteins. The concentrated β- and γ-globulin fractions of serum showed a weak γ_{tr}-line, but no evidence for presence of β_{tr}-protein.

Ouchterlony plates with serum, plasma and unconcentrated CSF surrounding "absorbed" anti-CSF in the center wall showed formation of a single line, weaker and closer to the antigen wells for 6 individual sera and 2 plasma samples studied than for the 6 individual unconcentrated CSF samples, indicating a lower concentration of the γ_{tr}-protein in serum



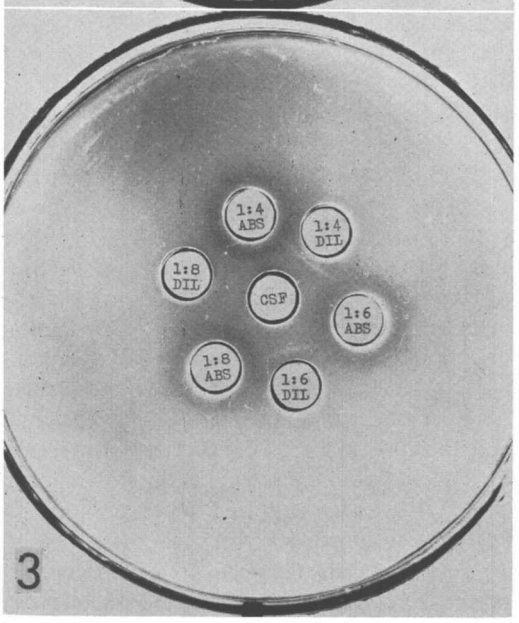


FIG. 2. Double diffusion in agar with an "absorbed" antiserum to CSF (Ab-A-CSF), specific for γ_{tr}-protein, in the center well; ASC, ascitic fluid; AC-PH, acute phase serum; N-H plasma; CSF-I; normal CSF; CSF-II and serum II, from a patient with a glioma. There is a line of identity demonstrating the presence of γ_{tr}-protein in each of these fluids.

FIG. 3. Double diffusion in agar of "absorbed" anti-CSF, specific for γ_{tr}-protein, after further dilution with serum or saline. ABS, dilution with serum; DIL, corresponding dilution with saline; CSF, unconcentrated cerebrospinal fluid. Dilution of "absorbed" anti-CSF with serum (1:6) fails to form a line, while a 1:8 dilution with saline shows a faint line.

than in CSF (Fig. 2). This line was also developed by the "absorbed" antiserum specific for γ_{tr}-protein. Urinary proteins precipitated by $(NH_4)_2SO_4$ did not develop the γ_{tr}-line whereas the urinary proteins concentrated to a higher degree by vacuum dialysis did.

"Absorbed" anti-CSF after dilution with saline up to 1:8 still formed the γ_{tr}-precipitation line in Ouchterlony plates with unconcentrated CSF. However, when the "absorbed" anti-CSF was diluted with human serum, a 1:6 dilution failed to form this line. This shows that further absorption with serum removes the antibody to γ_{tr}-protein (Fig. 3).

Discussion. The present results show that the preparation of antiserum to CSF has led to the immunological detection of trace proteins present in various biological fluids including CSF, plasma, ascitic fluid, urine, and pleural fluid. The presence of both proteins, β_{tr}- and γ_{tr}- was demonstrated in CSF, urine, and in one of the 2 ascitic fluids studies. Only γ-protein was found in pleural fluid and in serum in which cases the apparent absence of β_{tr}-protein was possibly due to the relatively low sensitivity of the methods used.

In view of the fact that these proteins are apparently not specific for CSF, as was proposed by Clausen(6), and since it is necessary to concentrate biological fluids for their immunoelectrophoretic detection, it would seem to be more appropriate to refer to them as γ_{trace}- and β_{trace}-proteins rather than γ_{CSF} and β_{CSF}(6).

More accurate measurements of their concentration in the various fluids are needed, but the present studies indicate that the concentration of γ_{tr}- in CSF is somewhat higher than in plasma. The apparent ease with which these proteins cross the blood-brain barrier and mesothelial linings may be indicative of a low molecular weight. Preliminary studies in this laboratory conducted in cooperation with Dr. E. C. Franklin point to the possibility that the β_{tr}- and γ_{tr}- proteins have sedimentation constants similar to the proteins described by Press(13). In fact, it seems likely that the proteins of β- and γ-electrophoretic mobility and of approximate

sedimentation constants of S = 2, found by Press(13) in concentrated spinal fluid, are identical to the β_{tr}- and γ_{tr}-proteins.

The antigenicity of these proteins can hardly be questioned. Although immunization with serum or plasma apparently does not result in antibodies to the trace components, this may be due to the relatively low concentration of these as compared to the other serum proteins. Evidence that γ_{tr}-protein cross-reacts with gamma globulin has not been obtained. The fact that anti-human serum or anti-human gamma globulin do not demonstrate this protein in immunoelectrophoresis, makes it doubtful that γ_{tr}-protein is identical with previously described proteins of similar electrophoretic mobility, present in serum(14) and urine(15) and immunologically related to gamma globulin.

Preliminary investigations in this laboratory indicate that antiserum specific for γ_{tr}-protein can give passive cutaneous anaphylaxis in guinea pigs, and that absorption with serum can neutralize this antibody activity. This further demonstrates that the observed precipitation lines in Ouchterlony plates and immunoelectrophoresis indeed represent antigen-antibody interaction.

The electrophoretic mobilities of β_{tr}- and γ_{tr}-proteins are similar in starch block and agar gel. The γ_{tr}- seems much less heterogenous in electrophoretic mobility than the β_{tr}-protein. The electrophoretic heterogeneity of the β_{tr}-protein was similar in all individual samples tested, whereas the γ_{tr}-protein varied slightly. At present, no further characteristic properties of these proteins have been found. Staining reactions for lipid or carbohydrate content and for oxidase or peroxidase activity were negative. The trace proteins do not bind radioactive iron. Attempts to further characterize these trace components and to determine their site of synthesis are in progress.

Summary. Rabbit antisera to human cerebrospinal fluid, after "absorption" with human serum, have been shown to react with various human biological fluids. The reactions have been studied by immunoelectrophoresis and double diffusion in agar, and have revealed the presence of 2 trace proteins of β and γ electrophoretic mobility. Both proteins, "β_{trace}"- and "γ_{trace}"-, were found in CSF, urine and ascitic fluid, while the γ_{trace}-protein was also demonstrated in serum and pleural fluid. Exhaustive absorption of the antiserum to CSF with human serum removed the antibody to the γ_{trace}-protein.

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