M.Sc. Experimental Medecine

ABSTRACT

by

Francois Feldmuller

ISOLATION AND PURIFICATION OF A BRAIN PROTEIN

Methods have been developed for the isolation of the two molecular sizes of the γ_C-globulin from bovine CSF, bovine colostrum and human CSF. These fluids were concentrated in Visking dialysis tubing under pressure and the filtrates concentrated by ultrafiltration. While γ_C-globulins were the only antigens in the Visking tubing (Vt) filtrates that could be detected with anti-CSF sera, three other proteins were revealed by disc gel electrophoresis. The smaller form of the colostral γ_C-globulin was separated from these impurities by filtration through Sephadex G-75. Its molecular weight was found to be about 15,000 by gel filtration. The larger γ_C-globulin was isolated from bovine CSF retained in the dialysis tubing by ammonium sulfate precipitation and chromatography on Sephadex G-75. Purification of the larger γ_C-globulin from colostrum required successive filtrations through Sephadex gels of increasing porosity. The molecular size was found to be 30,000 by gel filtration.

Short Title:

ISOLATION AND PURIFICATION OF A BRAIN PROTEIN

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by

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
EAE	experimental allergic encephalomyelitis
CSF	cerebrospinal fluid
IgM	Immunoglobulin M
IgA	Immunoglobulin A
IgG	Immunoglobulin G
TMED	N, N, N^1, N^1 - tetramethylethylenediamine
AP	ammonium persulfate
µg	microgram(s)
µl	microliter(s)
mA	milliampere(s)
mM	millimolar
cm	centimeter(s)
g	gram(s)
M	molar
mg	milligram(s)
ml	milliliter(s)
min	minute(s)
Tris	Tris (hydroxymethyl) aminomethane
DEAE-cellulose	diethylaminoethyl-cellulose
CFA	complete Freund's adjuvant
Vt-filtrate	Visking tubing filtrate

DEFINITION OF TERMS

It may be helpful to define a number of terms that will be used frequently.

An antigen is any substance which is capable, under appropriate conditions, of inducing the formation of antibodies and of reacting specifically, in some detectable manner, with the antibodies so induced.

The terms antibodies refer to proteins which are formed in response to the administration of an antigen and which react specifically with that antigen, to a variable extent, with substances of similar chemical structure.

Hapten is a term used to describe a molecule capable of reacting with an antibody but lacking the ability to induce formation.

The term immunogen is often used in referring to the substance that stimulates the formation of the corresponding antibody.

Sera that contain antibodies that appeared after the injection of antigens are called immune sera or antisera.

Reactions between an antibody and the inciting antigen are said to be homologous, while reactions taking place with substances other than the inciting antigen are called heterologous.

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GENERAL INTRODUCTION

1. Cerebrospinal Fluid:

The cerebrospinal fluid (CSF) is a clear, colorless fluid which fills the ventricles and subarachnoid spaces of the brain and spinal cord and may be regarded for the most part as an ultrafiltrate of the plasma produced by the choroid plexuses acting essentially as semipermeable membranes.

CSF and blood serum contain similar concentrations of inorganic salts and organic substances of small molecular weight but the very low concentration of protein in the CSF has been compensated for by increased amounts of chloride ion in order to satisfy the requirements of the Gibbs-Donnan equilibrium. Normal adult CSF contains from 20-40 mg of total protein per 100 ml and all of the proteins of serum are present except those having a molecular weight in excess of 200,000. The protein composition determined by paper electrophoresis is : prealbumin, 0-4.4% ; albumin, 51.3-60.0% ; α_1-globulin, 4.7-8.0% ; α_2-globulin, 7.5-11.6% ; β-globulin, 9.2-18.0% ; fibrinogen, 0-8.0% ; γ-globulin, 5.6-10.0%. Other organic constituents include amino acids, glucose, peptides, fatty acids, neuramanic acids, histamine, vitamins, and numerous enzymes such as cholinesterase and pseudocholinesterase.

The CSF of newborn infants has a high protein content (60 to 90 mg of total protein per 100 ml) presumably because of a poorly developed blood brain barrier. Soon after birth the level drops to 20 to 40 mg of protein per 100 ml where it remains until after middle age when it increases slowly with advancing age.

The precise origin of CSF is still in doubt but the majority of its water and electrolytes originate from the choroid plexuses which are located in the roof of each one of the four ventricles. The pH is between 7.4 and 7.6. In the adult human, from one-half to one litre of CSF is formed daily and in most species of mammals, about 0.5% of the total CSF volume is secreted per minute.

After formation, the CSF circulates from the lateral ventricles to the third ventricle, then through the aqueduct of Sylvius into the fourth ventricle. It then enters the subarachnoid space through the median and lateral apertures of the fourth ventricle and circulates around the brain and spinal cord before it is absorbed. The total volume of CSF in the human is about 150 ml.

The function of CSF is not known. The CSF is often considered to be a closed hydraulic system in which the brain is submerged. By being immersed in a fluid the brain is thereby relieved of the many stresses of gravitation and acceleration and provided with a mechanical buffer against the bony skull. Today, in addition, another function referred to as the "sink action of CSF" is postulated. The CSF is considered to be a low concentration compartment or a "sink" in close contact with the brain extracellular space resulting in a diffusion of solutes down a concentration gradient into the CSF. These solutes are then returned to blood by bulk flow.

There is no known chemical constituent of the CSF which serves a special function in the nervous system. In disease an increase in the total proteins is the most common biochemical abnormality. A high total protein may be due to a variety of patho-

logical conditions involving either the meninges, the brain or spinal cord. Electrophoretic analysis of the CSF proteins has become a very important diagnostic tool in clinical neurology.

II. LITERATURE REVIEW

Examination of the proteins in CSF has proved to be an important aid in the diagnosis of some neurological diseases. A study of the protein components in CSF by free electrophoresis and their relationship to the serum proteins was undertaken by Kabat, Moore and Landor (1). They found that the electrophoretic pattern of CSF resembled that of serum and that, in general, alterations in the profile of the serum proteins were reflected in the pattern of the CSF. In neurosyphilis and multiple sclerosis, however, an elevated level of gamma globulin occurred in the CSF but not in the serum.

Kabat, Glusman and Knaub (2) used an immunochemical method to measure the concentrations of gamma globulin and albumin in CSF because the large quantities of CSF needed for electrophoretic analysis precluded the use of this technique as a routine procedure. They showed that there was an increase in the absolute amount as well as in the percentage distribution of gamma globulin in the CSF in neurosyphilis and in multiple sclerosis.

Free electrophoresis has long since been replaced by zone electrophoresis and the supporting materials may be agar gel, cellulose acetate strips, or acrylamide gels. However, to apply all variations of the electrophoretic technique, preliminary concentration of CSF is required.

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Scheiffarth et al. (9) extended immunoelectrophoretic investigations to include CSF from patients suffering from different neurological diseases and brain tumours. They found no abnormalities in the patterns of CSF from these patients except that the naturally occurring prealbumin was absent. It was also pointed out in this study that the τ-fraction seen in paper and agar gel electrophoretic patterns of CSF corresponded to the slowly migrating part of the double-bowed precipitation line of transferrin.

In the CSF of patients with multiple sclerosis, syphilis and purulent meningitis, Frick (10) found that the increase in γ-globulin concentration appeared as a heavy precipitate along its whole length. In some cases a second γ-globulin arc was seen which fused with the rapidly migrating anodic part of the γ-globulin. In a few cases of purulent meningitis he noticed an increase in the number of lines in the alpha and beta globulin regions. The characteristic β-globulin precipitate which had not been identified at that time as transferrin, could not be demonstrated in patients in whom serum had passed into the CSF spaces.

Burtin (11, 12, 13) confirmed that normal CSF contained 1 prealbumin fraction, albumin, α_1-mucoprotein and α_1-glycoprotein and 2 to 3 proteins with an electrophoretic mobility in the α_2 range. However, he could could not demonstrate α_2-macroglobulin, ceruloplasmin and haptoglobin. One of the two β_1-globulins, one which formed a double line was identified as transferrin. The immunoelectrophoretic pattern of the γ-globulin line was short and consisted of the cathodic part.

Patterns of CSF were also examined on the basis of their total protein concentration. The immunoelectrophoretic pattern was normal in patients with a normal or a slightly elevated total protein concentration. Samples that had an elevated total protein could be divided into two groups. The first group exhibited a normal pattern while the second group showed qualitative changes in the protein pattern. With the exception of α_2-macro and γ-macroglobulin and β_1-lipoprotein, most of the serum proteins were detected. The increased γ-globulin concentration was shown by the greater density and length of the precipitin arc.

Since then several authors have carried out immunoelectrophoretic studies of CSF proteins using antisera prepared against normal human serum or against different human serum proteins (Clausen (14), Dencker and Swahn (15), Clausen, Kroogsgard and Quaade (16), Laterre, Heremans and Demanet (17), Ursing (18)). These authors found that in patients with different neurological diseases the CSF often contained one or more serum proteins that are not usually demonstrable in normal CSF but at the same time no pattern characteristic of a particular neurological disease has been found.

Clausen (14) working with both horse and rabbit antisera to normal human serum and with monospecific antisera against selected serum proteins, counted 16 precipitin arcs in the pattern of CSF that had been concentrated 1000 times. In addition to all previously known proteins, haptoglobin, ceruloplasmin, α_2-macroglobulin, β_1-A-C and γ_1-A-globulin were identified. There was an increase of the α_2-macro-

globulin and β_1-lipoprotein in CSF withdrawn from patients who had neurological abnormalities involving a "breakdown" of the blood-brain barrier.

Before 1961, all the proteins in CSF could be detected in the serum. Then Clausen (19) immunized rabbits with human CSF and found that the immunoelectrophoretic pattern of concentrated CSF contained two precipitin arcs that did not appear in comparable patterns of serum. At the same time, MacPherson and Cosgrove (20) reported finding a γ-globulin in CSF which corresponded to one of the proteins discovered by Clausen. The precipitin lines corresponding to the β and γ globulins were not affected when the anti-CSF sera were completely absorbed with human serum. The presence of these globulins in CSF has since been confirmed by several investigators (Hochwald and Thorbecke (21), Laterre and Heremans (22), Frick (23), Link (24), Heitmann and Uhlenbruck (25).

MacPherson (26) and Clausen (19) called the globulins γ_{csf}-globulin and β_{csf}-globulin and after 1963, MacPherson shortened the name of the γ-globulin to "γ_c-globulin". The precipitate arc corresponding to the β_c-globulin usually extended from the α_2-region to the middle of the γ-region, while the double arc formed by the γ_c-globulin could often be traced from the α_2-region to the far γ-area. Other research-workers sometimes referred to the 2 proteins found in CSF as β-trace and γ-trace globulins. MacPherson et al. (20) suggested that the usual position of the γ_c-globulin arc in immunoelectrophoretic patterns of CSF indicated that the protein migrated more slowly

than the IgG globulin and that the position of its precipitin arc close to the antiserum trench suggested it to be a smaller, more rapidly diffusing molecule than the IgG globulin. Usually the γ_c-globulin forms a double arc in immunoelectrophoretic patterns, indicating that in most samples of CSF the protein exists in two molecular forms having different electrophoretic mobilities.

MacPherson and Cosgrove (20) used a rabbit antiserum against CSF from patients with multiple sclerosis in an attempt to ascertain whether the CSF in these patients contained proteins not occurring in the CSF of healthy persons or in persons with other neurological diseases. With rabbit anti-CSF antisera, they were unable to find proteins characteristic of multiple sclerosis in the CSF from patients with this disease.

Hochwald and Thorbecke (27) used immunoelectrophoretic techniques and demonstrated that the γ_c-globulin existed in human urine, colostrum, pleural effusions and plasma fractions concentrated by starch block electrophoresis. The protein was detected in human brain extracts and in urine but not in extracts from the kidney by Laterre, Heremans and Carbonara (28).

MacPherson (26) undertook to isolate the human γ_c-globulin in pure form so that the concentration of this protein in health and during varied neurological illnesses could be measured by quantitative immunochemical methods. The initial purification procedure involved chromatography of CSF on DEAE-cellulose employing 0.05 M Tris-HCL

buffer at pH 8.5. The bulk of the CSF proteins were absorbed on the column and the γ_c-globulin was collected in the effluent that passed through the column. The eluate from this initial step contained the γ_c- and β_c-globulin and the IgG globulin. The purified γ_c-globulin was obtained by rechromatographing this solution on DEAE-cellulose. The average concentration of the γ_c-globulin in normal CSF was found to be approximately 1.0 mg per 100 ml by quantitative immunochemical measurements. In contrast, in multiple sclerosis and other demyelinating diseases, the concentration of the protein in CSF was on the average 30 per cent lower than normal.

The source of the protein is of interest because it has been shown to comprise about 5% of the total CSF protein in normal adults, and a higher percentage in infants. The normal ratio of the total protein concentration of CSF to that of serum is about 1:300 and the electrophoretic patterns of concentrated CSF are similar to those of serum. However, if the γ_c-globulin entered the CSF by transudation from the blood as the other CSF proteins do, the protein would not be found in CSF since there is rarely enough of it to be detected in serum. Hence MacPherson (29) suggested that the γ_c-globulin is secreted into the CSF by unknown cells in the brain and eventually enters the blood when the CSF flows through the valves in the tubules of the arachnoid villi.

In the hope of accelerating and facilitating the acquisition of data on the properties and functions of the human γ_c-globulin, other mammalian species were examined to see if they contained a comparable protein because progress in the study of the human protein was severely

hampered because of the small amounts of CSF available.

MacPherson and Saffran (30) found that bovine CSF resembles human CSF in containing a γ_c-globulin analogous to the human γ_c-globulin that also exists in two molecular forms with different electrophoretic mobilities but the same immunological specificity. Bovine CSF was also shown to contain a characteristic albumin but to lack a globulin comparable to the β_c-globulin of human CSF. These authors also showed that there were no detectable changes in the antigenic determinants of the characteristic γ_c-globulin with maturation, for the γ_c-globulin precipitin line of bovine calf CSF fused completely with the precipitin line of adult bovine CSF.

Changes in the γ_c-globulin concentration of bovine cerebrospinal fluid with maturation was studied by MacPherson (31). The average concentration in bovine CSF withdrawn from newly-born colostrum deprived calves was found to be 1.4 mg per 100 ml. This value dropped to 0.44 mg per 100 ml for animals approximately 4 months old (26 samples), while the average values for adult steers was half this amount, namely 0.22 mg per 100 ml.

The white and grey matter of the cerebrum, the cerebellum and the spinal cord of a 4-month-old calf were analyzed. The spinal cord and cerebral white matter contained 0.01 mg of γ_c-globulin per gm of wet tissue, while the cerebral grey matter and the cerebellum contained 0.003 mg per gm of wet tissue. The protein is apparently distributed throughout the tissue of the brain and spinal cord.

Analyses of five samples of colostrum showed this fluid to have the highest average content of γ_c-globulin of all the fluids and

tissues examined, namely 15.0 mg per 100 ml.

Link (24) described a method of isolating the γ_c-globulin from human cerebrospinal fluid by fractionation of human CSF on Sephadex G-200 and Sephadex G-75. From these gel filtration experiments, it was shown that the γ_c-globulin was smaller than serum albumin.

MacPherson and Feldmuller (32) devised a method for isolation of the γ_c-globulin from bovine CSF in immunologically pure form. Instead of column chromatography on DEAE-cellulose, ammonium sulfate precipitation was employed to remove the serum albumin. The globulin precipitate of bovine CSF was then separated on a Sephadex G-75 column into fractions one of which contained the pure γ_c-globulin. The protein was found to have a molecular size of about 30,000 daltons by gel filtration chromatography.

Colostrum, the initial secretion of the mammary gland following parturition, differs from milk in that it contains more mineral salts, more serum proteins, casein, and less lactose. The transition from colostrum to a fluid having the composition of normal milk is complete in about four days. It has been shown by Murphy, Aalund, Osebold and Carrol (33) and by Dixon, Weigle and Vazquez (34) that almost 90% of colostral whey protein is IgG globulin and in colostrum formed in the first few hours after parturition the concentration of IgG may be 100 times greater than that of serum albumin. The electrophoretic profile of the serum of a newly-born bovine calf indicates that it is almost devoid of IgG-

globulin. This deficiency is remedied as soon as the calf ingests colostrum, for at birth the gut of the calf is permeable to serum proteins for the first day of life.

MacPherson (31) has shown colostrum to have the highest concentration of the γ_c-globulin of all biological fluids studied. The level decreases rapidly after parturition and approaches that of milk in a few days.

MacPherson and Feldmuller (32) devised a method for the isolation of the bovine γ_c-globulin from colostrum by repeated chromatography on Sephadex G-75.

The purpose of the present research was to devise methods for the isolation in pure form of the γ_c-globulin of human and bovine CSF and to ascertain some of the physical and chemical properties of the protein. It is hoped that studies of this nature may eventually yield important information regarding the role that some cerebral proteins play in the function of the brain.

111. EXPERIMENTAL

A. MATERIALS:

Human cerebrospinal fluid (CSF) was collected from patients undergoing pneumoencephalography or lumbar punctures in the course of the routine investigations of their neurological illnesses at the Royal Victoria Hospital. The CSF was centrifuged, pooled and stored at -20°C until needed.

Normal bovine CSF was drawn at the slaughter house from 4- to 6-month old calves or from three-year-old steers about 15 to 20 minutes after they had been killed by exsanguination. The bovine CSF was either processed immediately or was stored at - 20°C after removal of cells by centrifugation.

Bovine brain : Calf brains were obtained within one hour of death, cut into one half inch slices and frozen at -20°C.

Human brain : The human brain tissue was received less than 12 hours after death from victims of accidents or heart attacks and were cut into 1/2-inch slices and the slices frozen separately at -20°C.

Bovine serum : Bovine blood from calves or adult animals was collected at the slaughter house from animals that had been killed by exsanguination.

Bovine colostrum : Colostrum was obtained during the first day after parturition through the courtesy of Dr. A. Lagacé of the Ecole de Médecine Vétérinaire de la Province de Québec, and from Dr. D. G. Ingram of the Ontario Veterinary College at the University of Guelph, Ontario. The fluids were frozen and stored until needed.

Sephadex G-75, Sephadex G-100 and Sephadex G-150, inert non-ionized polymers of glucose, and blue Dextran 2000, a high molecular weight dextran having an average weight of 2×10^6 and covalently bound to a blue dye, were manufactured by A. B. Pharmacia, Uppsala, Sweden.

Cytochrome c (horse heart), ribonuclease (bovine pancreas), α-chymotrypsinogen (bovine pancreas), and crystalline trypsin (bovine pancreas) were products of Sigma Chemical Company, St Louis, Missouri. Bovine serum albumin, Fraction V and bovine γ-globulin, Fraction II were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Cyanogum-41, ammonium persulfate (reagent grade), glycine (ammonia free) and sucrose (reagent grade) were bought from Fisher Scientific Co. N, N, N^1, N^1 - tetramethylethylendediamine was purchased from Eastman Organic Chemicals, Rochester, New York. Amido black-10B was obtained from E. Merck Ag., Darmstadt, Germany.

The Visking dialysis tubing was manufactured by Union Carbide of Canada Limited ; the collodion membranes used for concentration of CSF by positive pressure dialysis were obtained from Membranfiltergesellschaft, Goettingen, Germany.

Crystalline rennin was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, while commercial rennin preparations known as " Rennet " tablets were obtained from Salada Foods Ltd., Toronto, Canada.

B. METHODS

I. Anti-sera :

Anti-bovine CSF : Rabbits were immunized with pooled calf CSF, containing about 25 mg of total protein per ml. One ml of the concentrated CSF was emulsified with an equal amount of complete Difco Freund's adjuvant (CFA), and about 0.30 ml of the emulsion was injected into six sites in the hind legs of each rabbit. One month after injection the animals were bled from the marginal ear vein, reinjected as above, and bled 3 weeks following the second injection. Thereafter, rabbits which produced sera with a high level of antibodies were immunized and bled regularly at approximately monthly intervals. The antisera were stored at -20°C. When in use, the sera were preserved with phenol (0.25%) and kept as much as possible at +4°C.

Anti-human CSF : Rabbit serum against human CSF was prepared as described by MacPherson (20).

Anti-bovine IgG : The same method as described above for anti-bovine CSF was used to prepare antisera to bovine IgG globulin, Fraction II. Each animal was injected with 5 mg of bovine IgG globulin.

Anti-bovine γ_c-globulin : Antisera to purified γ_c-globulin were prepared by immunizing each rabbit with 0.50 mg of purified γ_c-globulin emulsified in CFA. Some of the γ_c-globulin preparations that were used contained traces of IgG. The antibody that was formed against the IgG globulin was absorbed at the equivalence point with pure bovine IgG globulin, so that only the antibody to the γ_c-globulin remained in the

anti-γ_c-globulin sera.

Absorption of antisera : Anti-CSF sera were made specific for proteins characteristic of CSF by adding 0.05 ml of calf serum or calf plasma to 1.0 ml of anti-CSF serum. After mixing, the sera were allowed to stand at 37°C for one half hour, and the specific precipitate was removed by centrifugation at 1,500 rpm. The process was repeated with the supernatant as many times as necessary to obtain a supernatant that did not form a precipitate with a fresh addition of serum. Usually two absorptions were sufficient to remove all antibodies to serum proteins.

2. BOVINE BRAIN EXTRACTS:

Frozen bovine brain extracts were homogenized at 0°-4°C with a Tri-R homogenizer set at half full load speed for 2 min. Four (4) ml of cold saline were used for each gram of brain slice. The homogenates were centrifuged for one hour at 50,000 x g in a Spinco Model L preparative ultracentrifuge. The clear supernatants were removed and the precipitates were resuspended in half the original volume of cold saline and centrifuged again. The supernatants were combined and concentrated by positive pressure dialysis (35) at 4°C to the desired protein concentration, normally about 30 mg per ml. These were stored at -20°C.

3. HUMAN BRAIN EXTRACTS:

When extracts of this type were needed, the frozen tissues were processed as described for bovine brain.

4. BOVINE SERUM:

Bovine blood from calves or adult animals was allowed to clot at room temperature for 1 hour and the serum was collected after centrifugation at 1,500 rpm. Usually samples from ten animals were pooled and the serum stored in the frozen state at -20°C until needed.

5. PREPARATION AND CONCENTRATION OF CSF:

Pooled bovine CSF was filtered through a plug of glass wool to remove pieces of fat and flesh and then centrifuged at 5,000 rpm, for 30 min at 0°C to remove cells. Enough sodium azide was added to the CSF to bring the concentration to 0.10%.

The CSF was next concentrated from one half to one third the original volume by rotary evaporation under partial vacuum. Partially concentrated CSF was then added to 6 ft of 1/4 inch diameter Visking dialysis tubing knotted at one end and fastened to a rubber stopper at the other end. The stem of a funnel passed through 2 stoppers, the larger of which fitted into the neck of a 4 litre aspirator bottle and the smaller of which fitted into the open end of the Visking dialysis tubing and was secured by elastic bands.

The assembly was placed in the aspirator bottle and a partial vacuum of 17 cm of mercury was applied for a period of 18 hours. At the end of this period the filtrate (150 - 200 ml) was collected. The concentrated CSF was added to a fresh length of tubing, and the process continued for another 18 hrs until the CSF retained in the

Visking dialysis tubing had a concentration of 15-20 mg of protein per ml. If more concentrated solutions were needed, concentration was continued in the collodion bags manufactured by Membranfilter-gesellschaft, Germany. The Visking tubing filtrates (Vt-filtrates) and the retained CSF proteins were examined for γ_c-globulin activity by gel diffusion tests with appropriate antisera. A diagram of the concentration assembly is shown in Diagram A on p. 19.

6. AMMONIUM SULFATE PRECIPITATION OF CSF:

Clear solutions of CSF containing 30 to 35 mg of protein per ml were adjusted to pH 7.5 with 0.1 M sodium hydroxide. A previously calculated volume of saturated ammonium sulfate was added dropwise with constant stirring until the concentration of the ammonium sulfate was brought to 60% saturation. The globulins were removed by centrifugation at 8,000 rpm at 0°C for 1 hour. The supernatant was poured off and the precipitate recentrifuged and the residual supernatant removed with a pasteur pipette and discarded. Entrained supernatant, containing chiefly serum albumin, was washed out of the packed precipitate by suspending the latter in finely divided form in a volume of 60% saturated ammonium sulfate solution equal to one third the starting volume. The precipitate was centrifuged as above and the washings were discarded. The precipitate was then dissolved to one half the original volume with water and a second precipitation and washing was carried out as described above. Finally, the precipitate

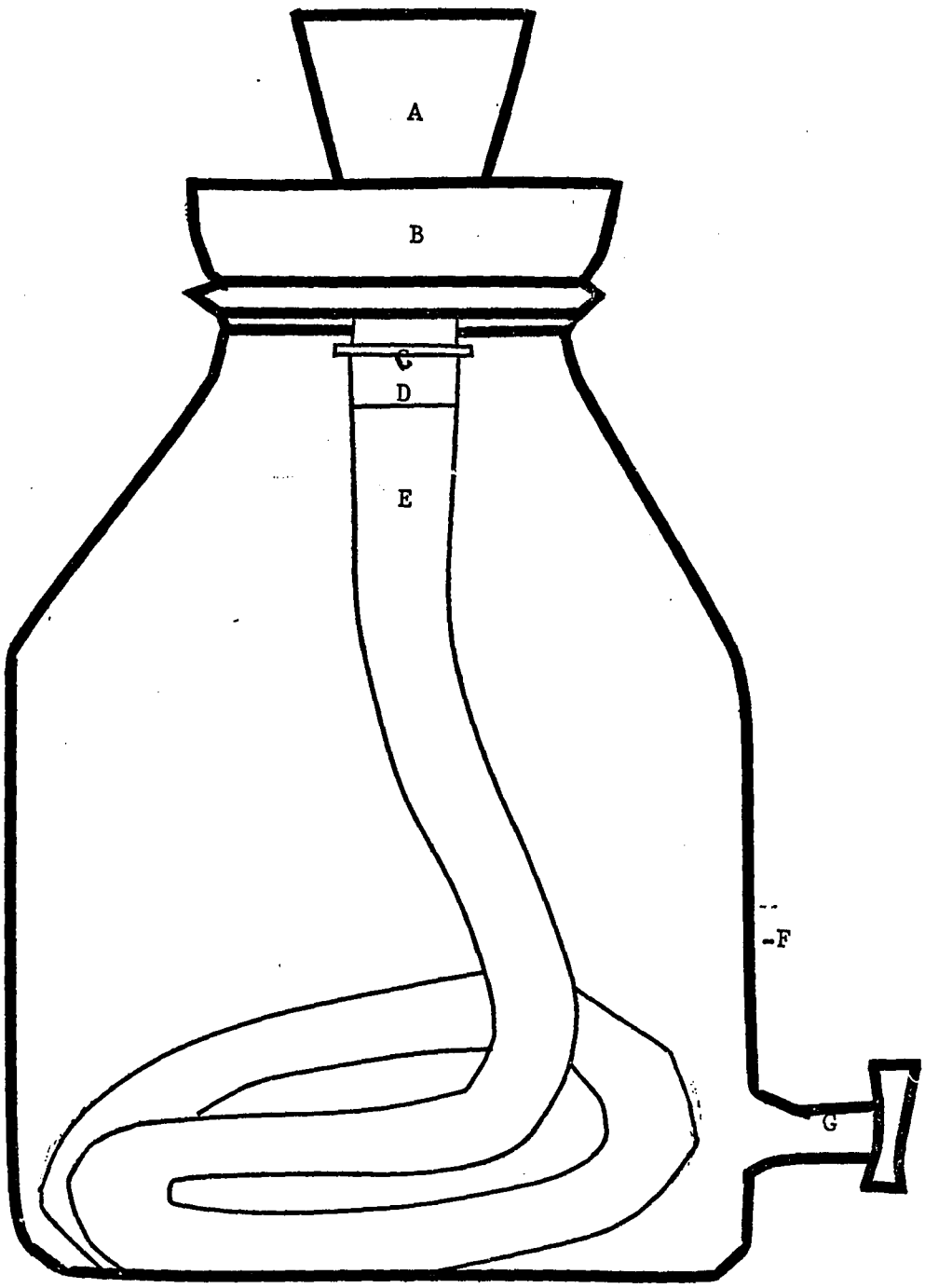

Diagram A: Diagram of the Concentration Assembly. A). Funnel ; B).Large rubber stopper ; C) Elastic ; D). Small rubber stopper ; E). Visking dialysis tubing ; F). Aspirator bottle ; G). Outlet

was dissolved in sufficient water to bring the concentration to 4 mg per ml. This solution was tested by gel diffusion against an anti-bovine CSF antiserum and an anti-bovine γ_C-globulin antiserum and was usually free from bovine serum albumin.

7. PREPARATION OF COLOSTRAL WHEY:

(a). With acetic acid. The defatted colostrum was warmed to 37°C and the pH adjusted carefully to the iso-electric region (4.0 to 4.8) with 3 M acetic acid until precipitation occurred. The precipitate was separated from the whey by centrifugation and the pH of the whey was then adjusted to pH 6.5 with 1 M sodium hydroxide. The lactoglobulin precipitate was removed by centrifugation, and the supernatant, or whey, was used for preparative chromatography.

(b). With crystalline rennin. The defatted colostrum was warmed to 37°C and then 5 ml of a solution of crystalline rennin (100 mg per ml) was added to 100 ml of colostrum with constant stirring. After the precipitate settled it was removed by centrifugation at 5,000 rpm.

(c). With commercial rennin. The casein was removed from the defatted colostrum by clotting with " Rennet " tablets. Approximately 6.4 ml of a solution containing 100 mg of " Rennet " tablets per 1.0 ml was used for every 100 ml of colostrum. The precipitate was removed by centrifugation.

8. GEL FILTRATION CHROMATOGRAPHY:

Sephadex G-75, Sephadex G-100 and Sephadex G-150 were allowed to

swell for 48 hrs in Elliott's solution (an artificial CSF containing the inorganic components of CSF but devoid of glucose or protein). Suspensions of the swollen gels were deaerated under reduced pressure before packing the columns. Each column was prepared by pouring a thin slurry of gel particles in Elliott's solution into chromatographic columns that were partly filled with the same solution. The addition of gel was continued until the desired bed height was reached. Elliott's solution was then passed through the column for 24 hrs before the sample was applied. In the early stage of this research 0.01 M phosphate buffer, pH 8.5, was used instead of Elliott's solution.

For the first crude preparative separation of colostral whey, 100 ml samples were applied to a 9 x 40 cm column packed with Sephadex G-75 and were run at a flow rate of 100 ml per hr. One hundred ml fractions were collected.

A 4 x 50 cm column packed with Sephadex G-75 was used for the fractionation of the globulin precipitates of human and bovine CSF and colostrum. The sample size for this column was 10.0 ml ; five ml fractions were collected. The flow rate was 30 ml per hour.

For Vt-filtrates 2 ml samples were applied to columns 2 x 80 cm packed with Sephadex G-75. The flow rate was 5 ml per hr ; one ml fractions were collected. The protein concentrations of the effuents were measured in a Zeiss spectrophotometer PMQ II at a wavelength of 280 mμ.

9. MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION:

A column of 2 x 80 cm was packed with Sephadex G-75 as described

above. The column was calibrated following the method of Andrews (36) using proteins of known molecular weight as standards. The proteins were dissolved separately in 1.0 ml of Elliott's solution at a concentration of 2 mg per ml and applied to the top of the Sephadex bed. The void volume of the column was determined with Blue Dextran. The effluent was collected in 1.0 ml fractions. The protein concentration of the effluents was measured in a Zeiss spectrophotometer PMQ II at 280 mμ. The concentration of cytochrome c was determined at 412 mμ. The maximum concentration of a protein in the effluent was estimated to the nearest 1 ml from the elution diagram.

10. DISC GEL ELECTROPHORESIS:

Polyacrylamide disc gel electrophoresis was performed according to the method described by Davis (37) with the following modifications: (1) the running gel solution was prepared by dissolving 14 g of Cyanogum-41, 0.20 ml of N, N, N^1, N^1 - tetramethylethylenediamine (TMED) and 0.02 ml of Tween-80 in 200 ml of 0.4 M Tris-HCL buffer pH 8.9. Ammonium persulfate (0.1 g) was added to the above solution just before filling the tubes. (2) the spacer gel solution was prepared by dissolving 4.0 g of Cyanogum-41 in 100 ml of 0.06 M Tris-HCL buffer, pH 6.7, containing 0.1 ml of N, N, N^1, N^1 - tetramethylethylenediamine (TMED) and 0.01 ml of Tween-80. Ammonium persulfate (1.0g) was added to the above solution just before filling the tubes. The protein sample (50 - 200 μg of protein) was dissolved in 0.1 ml of 0.06 M Tris-HCL buffer, pH 6.7 containing 10% sucrose. The sample

was placed on top of the spacer gel and the electrode buffer was carefully layered over the sample. A constant current of 2.5 mA per tube was applied until the sample dye was in the spacer gel, then 3.0 mA per tube was applied until the Kohlrausch boundary had travelled 6 cm in the running gel. The tubes were placed in ice-water and when thoroughly chilled, the gels were forced out by applying a stream of compressed air to the open end of the running tube. The gels were stained with Amido Black-10B (1%) in acetic acid (7%) for 30 minutes and destained electrophoretically for 2 hours.

II. IMMUNOLOGICAL ANALYSES:

(a). DOUBLE DIFFUSION ANALYSIS: Ouchterlony type analyses were carried out on glass microscope slides. The slides were covered with agar (1%) dissolved in barbiturate buffer pH 8.3, 0.05 ionic strength containing sodium azide (0.1%) as a preservative. The wells were cut in the solidified gel according to a suitable pattern. The antigen solutions and the antisera were then placed in the appropriate wells. After the plates were incubated at room temperature for 12 to 24 hours, the slides were washed in several changes of saline for 24 tp 48 hrs, photographed when desired, dried, and then stained with 0.1% Azocarmine.

(b). IMMUNOELECTROPHORESIS: Immunoelectrophoresis was carried out according to the method described by Wieme and Rabaeye (38) on glass microscope slides prepared in the same manner as for double diffusion analyses. After electrophoresis had proceeded for 30 minutes

at a current of 40 mA per slide, about 0.25 ml of antiserum was added to a 2 mm trough parallel to the path of electrophoretic migration. The microelectrophoretic plates were incubated at room temperature for 12 to 24 hours. The slides were then washed with saline for 24 hrs, photographed, dried, and stained with 0.1% Azocarmine.

(c). DETERMINATION OF ANTIGEN CONCENTRATION BY DIFFUSION IN ANTI-BODY CONTAINING AGAR: This determination was carried out by the method of Fahey and McKelvey (39). Rabbit anti-γ_c-globulin serum was diluted with two volumes of 0.15 M phosphate buffer, pH 7.5, heated to 56°C and then mixed with an equal amount of a 3% solution of agarose in 0.15 M sodium chloride previously heated to 56°C. Eighteen ml of the well mixed antibody agar solution was poured onto glass plates (8 x 10 cm). Four standard solutions of pure γ_c-globulin of known protein content were added to wells on each plate. After 20 hrs at room temperature, the diameters of the rings were read with a pocket model Finescale comparator. Using semilog paper, the ring diameters in mm were plotted as the abscissae and the protein concentrations as the ordinates. The concentration of the γ_c-globulin in the various fluids was calculated from a standard line drawn by the points determined with the standard solutions of γ_c-globulin. The slides were washed with saline, dried and stained.

12. DETERMINATION OF PROTEIN CONTENT:

The protein concentration was determined by the method of Lowry

et al. (40) or by the sulfosalicylic method of Cipriani and Brophy (41). Either bovine serum albumin, Fraction V or bovine IgG globulin, Fraction II were used as standards in the Lowry method. A sample of dialyzed serum the protein of which had been calculated from the nitrogen content (determined by microKjeldahl) was the standard for the sulfosalicylic acid method.

IV. RESULTS

A. RECOVERY OF γ_c-GLOBULIN AFTER DIFFERENT METHODS OF REMOVING CASEIN:

It was shown earlier (31) that colostrum has the highest average content of γ_c-globulin of all the fluids and tissues that have been examined so far. In the preliminary stages of this research acetic acid was the only reagent used to remove casein by iso-electric precipitation. This treatment was found to have a detrimental effect on the immunochemical properties of the γ_c-globulin. Therefore it was important to find a method for removing casein which did not destroy the γ_c-globulin. Thus, casein was removed from 100 ml aliquots of a defatted colostrum solution in three different ways.

One hundred ml of defatted colostrum was taken and the pH adjusted carefully to the iso-electric region (4.0 - 4.8) with 1.5 ml of 3 M acetic acid. The precipitate was separated from the whey by centrifugation and the pH of the colostral whey was then adjusted to pH 6.5 with 1.8 ml of 1 M sodium hydroxide. The lactoglobulin precipitate was removed by centrifugation, and the supernatant was brought to the original volume (100 ml) with 0.15 M sodium chloride.

To 100 ml of the same defatted colostrum was added 5 ml of a solution of crystalline rennin (100 mg of rennin per ml). The clotted casein was removed by centrifugation and the supernatant brought to a final volume of 100 ml with 0.15 M sodium chloride.

The last method involved adding 6.4 ml of a commercial preparation of rennin (100 mg per ml) to 100 ml of the defatted colostrum. The

TABLE I

RECOVERY OF γ_c-GLOBULIN FROM COLOSTRUM AFTER REMOVAL OF CASEIN BY DIFFERENT METHODS

Colostrum	γ_c-globulin (mg /100 ml)	Recovery (%)
Untreated	15.5*	100
Treated with acetic acid	10.0*	64.0
Treated with commercial rennin	14.5*	93.5
Treated with crystalline rennin	12.5*	80.6

* Values were determined by the Fahey and McKelvey (39) antibody in agar method using an antiserum specific for the γ_c-globulin.

casein was removed as described above and the supernatant adjusted to a final volume of 100 ml. The results are shown in Table 1 on p. 27.

It will be seen that the highest recovery of the γ_c-globulin activity occurred when a commercial preparation of impure rennin was used and that the use of acetic acid at pH 4.0 destroyed about 40% of the γ_c-globulin. In all subsequent experiments, the commercial rennin was used for the removal of casein.

B. PREPARATIVE GEL-EXCLUSION CHROMATOGRAPHY OF COLOSTRAL WHEY:

One hundred and twenty five to one hundred and fifty ml of colostral whey (total protein 2,800 mg) were applied to a Sephadex G-75 column (9 x 40 cm) and eluted with Elliott's solution. One hundred ml fractions were collected per hour manually. The elution pattern is shown in Figure 1 on p. 29.

Examination of the elution profile showed a band of brownish pigment (noted during the fractionation) was eluted in fractions 1,000 through 1,400 ml and subsequently these fractions were found to contain the highest protein concentrations. A band of yellowish pigment was found in fractions eluted between 3,000 to 3,900 ml. To locate the γ_c-globulin activity, a 3 ml sample of each fraction was taken and concentrated to 0.1 ml by positive pressure dialysis. The eluate collected between 0-100 ml is referred to as Fraction 1 ; similarly, that collected between

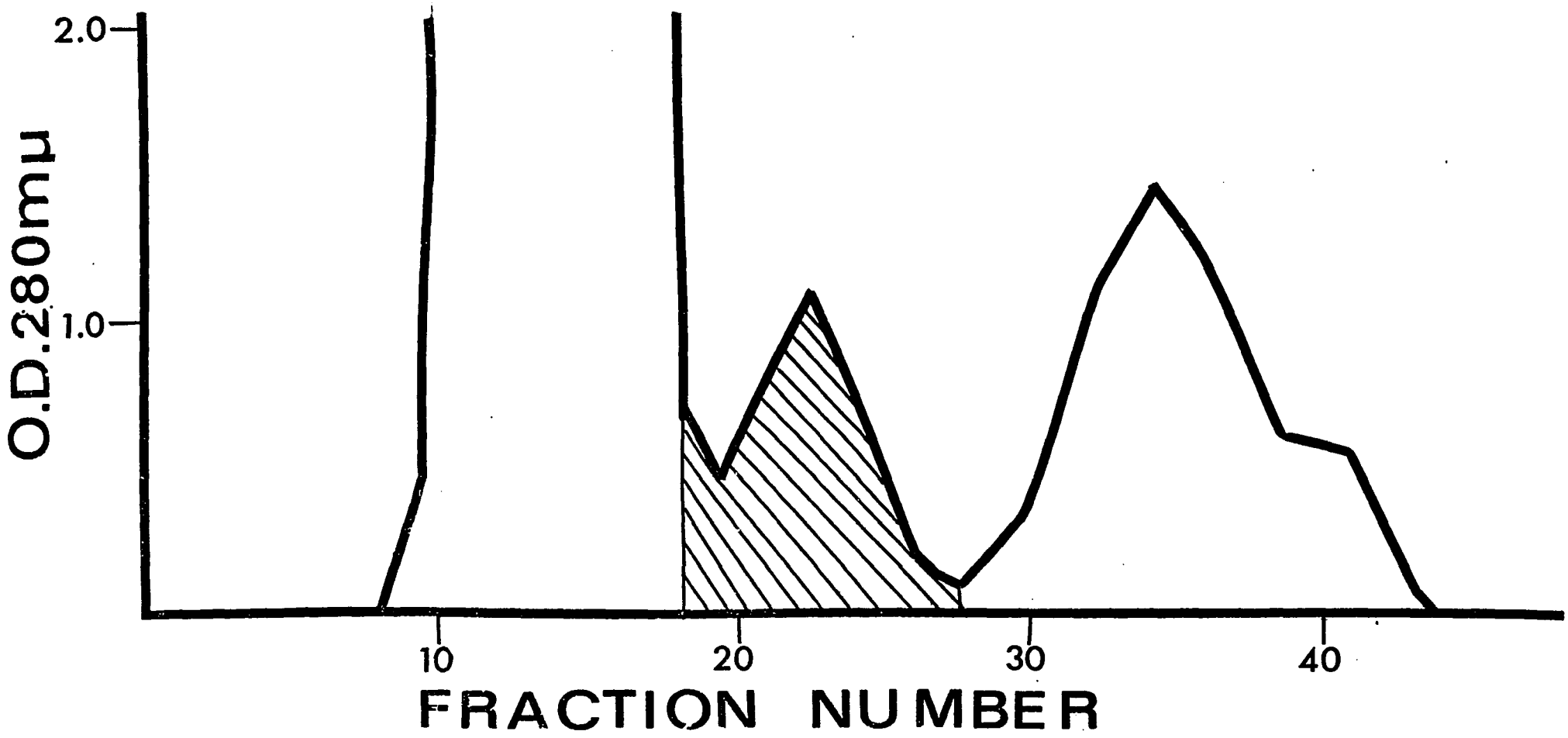

Figure 1 : Preparative gel-exclusion chromatography of colostral whey on Sephadex G-75.

100 - 200 is referred to as Fraction 2. The 40 fractions were analyzed by gel diffusion experiments for the γ_c-globulin content and results are shown in Figure 2 on p. 31. It will be seen that the γ_c-globulin is present in Fractions 19 to Fractions 27 and is thus eluted in a volume of 800 ml as shown by the shaded area on the chromatogram.

The protein responsible for the first and main peak of the elution diagram (Figure 1) is IgG. Almost 90% of colostral protein (33, 34)is IgG and most of this can be separated from the γ_c-globulin by this first crude separation on Sephadex G-75. The fractions containing γ_c-globulin are of course heavily contaminated with IgG globulin. At this stage the γ_c-globulin was eluted in only one section of the chromatogram (shaded area), but it is not pure enough to draw any conclusion about its homogeneity in respect to size.

Fractions eluted between 1,800 and 2,200 ml (Fraction A) and between 2,200 and 2,600 ml (Fraction B) were combined and 2 ml samples of the two were concentrated to 0.1 ml by positive pressure dialysis in order that each of them could be analyzed by immunoelectrophoresis. The results of these analyses are shown in figure 3 and Figure 4. Both immunoelectrophoretic determinations were carried out with an antiserum to bovine CSF. This antiserum will detect all antigenic proteins in CSF. It can be seen from both figures (3 and 4) that the colostral whey contains a number of

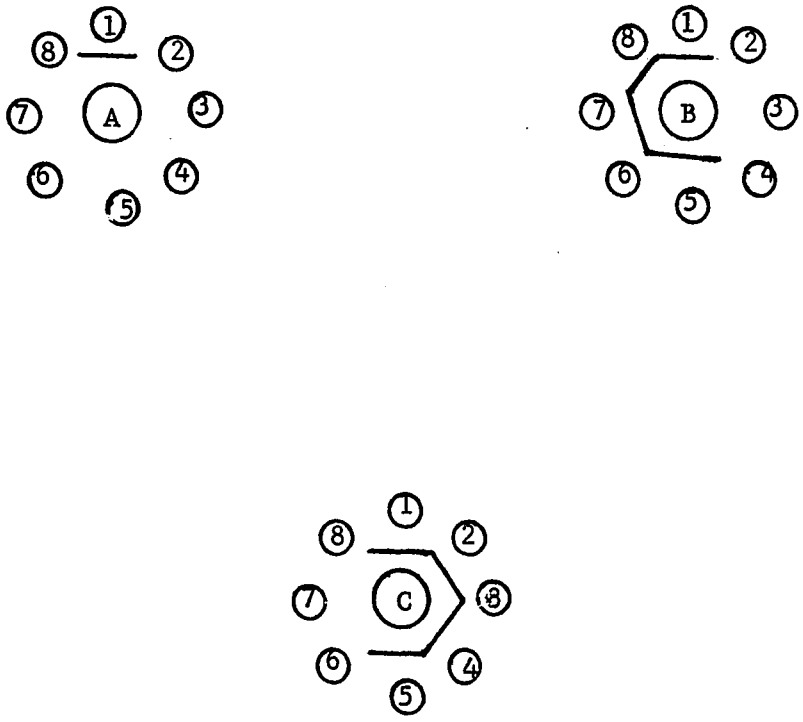

Figure 2. Double diffusion analysis of effluent from gel filtration chromatography of colostral whey on Sephadex G-75. The center wells A, B and C contained a rabbit antiserum specific for the γ_C-globulin. The peripheral wells were filled with the effluent fractions recorded below.

A. 1. Original colostrum; 2. Fraction 9 ; 3. Fraction 10; 4. Fraction 11; 5. Fraction 12; 6. Fraction 13; 7. Fraction 14; 8. Fraction 15.

B. 1. Original colostrum; 2. Fraction 16; 3. Fraction 17; 4. Fraction 18; 5. Fraction 19; 6. Fraction 20 ; 7. Fraction 21; 8. Fraction 22.

C. 1. Original colostrum; 2. Fraction 23; 3. Fraction 24; 4. Fraction 25; 5. Fraction 26; 6. Fraction 27; 7. Fraction 28; 8. Fraction 29.

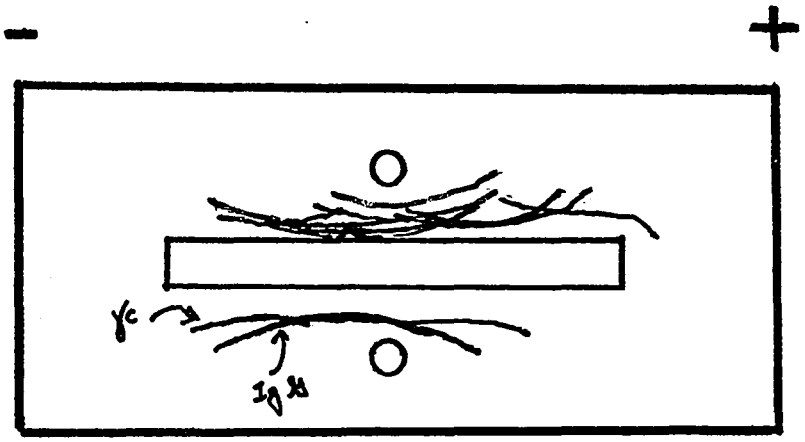

Figure 3: Immunoelectrophoretic analysis of untreated colostrum (upper well) and material eluted between 1800 to 2200 (Fraction A) ml developed with a rabbit anti-bovine CSF serum.

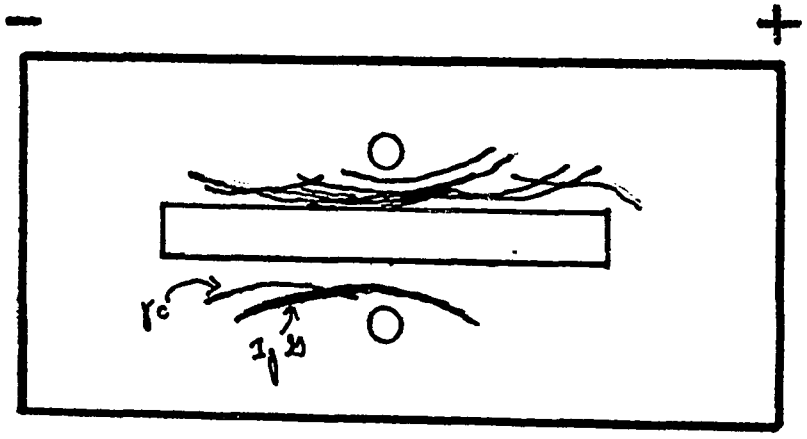

Figure 4: Immunoelectrophoretic analysis of untreated colostrum (upper well) and material eluted between 2200 to 2600 (Fraction B) ml (lower well) developed with a rabbit anti-bovine CSF serum.

proteins ranging from gamma-globulins to albumins. In contrast, however, Fraction A contains three (3) components; IgG, γ_c-globulin and a protein having a mobility in the α- to β- range. In the immunoelectrophoretic pattern of Fraction B, there are only two antigenic proteins, the γ_c-globulin and the IgG globulin.

When the immunoelectrophoretic analyses were carried out with antiserum specific for the γ_c-globulin, the following results (see Figure 5 and 6) were obtained.

It will be noted that Fraction A contained much more protein than Fraction B. In practice, several lots of Fraction A and B were combined, concentrated and rechromatographed to remove the contaminating globulins.

C. PURIFICATION OF ELUATES (FRACTION A AND B) CONTAINING THE γ_c-GLOBULIN:

Concentration in Visking dialysis tubing under pressure (see methods) was employed to reduce the eluates containing the γ_c-globulin to their original volume because the protein content of the solutions was too high to use rotary evaporation. On the other hand, the Vt-filtrates contained very little protein and they were reduced to their original volume by rotary evaporation.

When the concentrated Vt-filtrates were tested with a rabbit anti-bovine CSF antiserum and a rabbit anti-γ_c-globulin antiserum, it

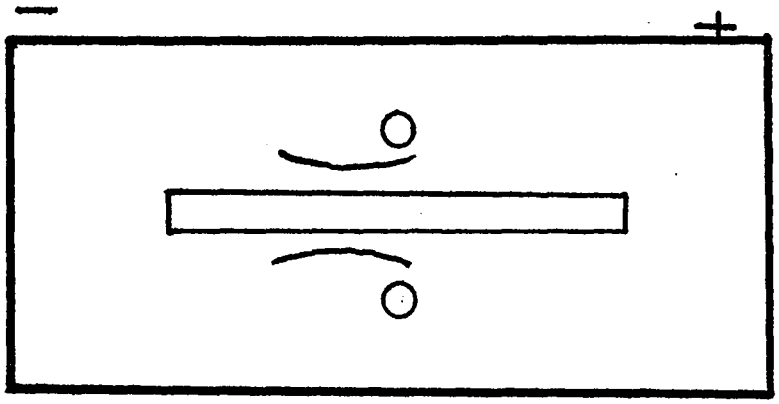

Figure 5: Immunoelectrophoretic analysis of colostral whey (upper well) and material eluted between 1800 to 2200 ml Fraction A in lower well, developed with an antiserum specific for the γ_c-globulin.

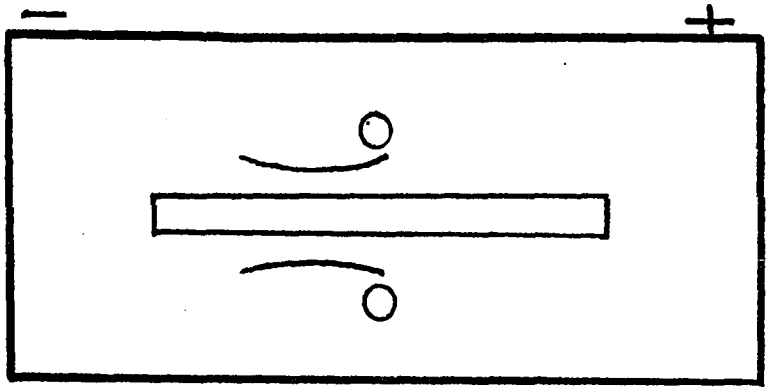

Figure 6: Immunoelectrophoretic analysis of colostral whey (upper well) and material eluted between 2200 to 2600 ml Fraction B in lower well, developed with an antiserum specific for the γ_c-globulin.

was found that these Vt-filtrates contained a substance with the immunochemical properties of the γ_C-globulin. The reults are shown in Figure 7. The precipitin arcsformed by the Vt-filtrates do not fuse with the arc formed by the standard bovine IgG in well numbers 2 and 6 or with the band formed by the standard BSA opposite well numbers 4 and 8, indicating that the Vt-filtrates contained the γ_C-globulin, free from IgG and BSA.

Each concentrate of Vt-filtrate formed one (1) band with a rabbit anti-γ_C-globulin serum and all of them fused with each other as is shown in Figure 8. The fusing of the precipitin bands formed by the standard CSF and a standard colostrum with the precipitin bands of the four (4) Vt-filtrates indicate that these Vt-filtrates contained the γ_C-globulin.

At times it was noted that concentration of the Vt-filtrates by rotary evaporation resulted in precipitation of unknown substances during the process of concentration. Sometimes the precipitate would appear while the solution was being concentrated and sometimes it did not appear until the concentrated solutions were frozen. Frequently, the presence of the precipitate coincided with a substantial loss of the γ_C-globulin activity.

The precipitate was not soluble in 0.15 M saline or in 0.1 N sodium hydroxide but dissolved in 0.1 N HCL.

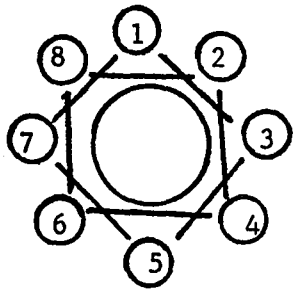

Figure 7: Double diffusion analysis of colostral Vt- filtrates wells numbered 1,3,5 and 7.Wells numbered 2 and 6 were filled with IgG and wells numbered 4 and 8 were filled with BSA. The center well contained a rabbit anti-bovine CSF serum.

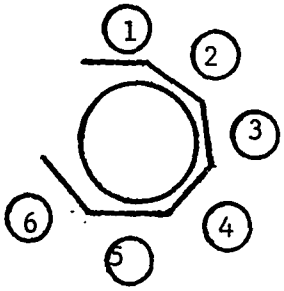

Figure 8: Double diffusion analysis of colostral Vt- filtrates wells numbered 3,4,5 and 6 and in well numbered 1 was filled with a standard bovine CSF (20 mg/ml) and well numbered 2 was filled with a a standard colostrum (15 mg/ml). The center well contained a rabbit anti-γ_c-globulin serum.

In order to avoid this deleterious build up in salt concentration and the consequent loss of γ_c-globulin activity, the Vt-filtrates were concentrated in collodion bags under positive pressure. Although this method took more time, the increased salt concentration and loss of γc-globulin was avoided. To ascertain whether some γ_c-globulin was able to pass through these smaller pored membranes, the filtrates were collected, concentrated by rotary evaporation and then tested with a rabbit anti-bovine CSF and a rabbit anti-γ_c-globulin sera. No γ_c-globulin was found in these filtrates from the collodion bags.

Two experiments were carried out to ascertain whether the impure form of commercial rennin used to remove the casein might have degraded the γ_c-globulin due to some unknown enzymes. One hundred ml of untreated colostrum were placed in Visking dialysis tubing and concentrated to 3/4 the original volume in the usual manner. The Vt-filtrate was concentrated and analyzed with anti-CSF and anti-γ_c-globulin sera. Both tests were positive, indicating that a small γ_c-globulin was present in untreated colostrum and was small enough to pass through the Visking tubing.

The second experiment involved diluting 100 ml of untreated colostrum three-fold with Elliott's solution and placed in Visking

tubing and concentrated in the usual manner. The Vt-filtrate was concentrated and found to contain the γ_c-globulin.

These results show that the use of the impure rennin to to clot casein in colostrum did not create the small γ_c-globulin molecule. If the rennin did split the γ_c-globulin, there should have been no evidence of the small protein in the Vt-filtrates when untreated colostral whey was concentrated.

To summarize, there is a molecular species of γ_c-globulin in the Vt-filtrates and when such Vt-filtrates are tested with the appropiate antisera, the γ_c-globulin seems to be immunologically pure. To analyze further the purity of the Vt-filtrates and to ascertain whether the electrophoretic mobility was changed by dilution or by treatment of colostrum with rennin an immunoelectrophoretic analysis was carried out as shown in Figure 9. In the electrophoretic pattern of untreated colostrum (upper well), there are about 10 to 12 precipitin bands which are distributed from the IgG area up to the albumin region. The Vt-filtrate pattern (bottom well) contains only one (1) precipitin band, indicating that the preparation of the γ_c-globulin in the Vt-filtrate is not contaminated with any antigens that are common to bovine sera, CSF and colostrum.

As is shown in Figure 10, the electrophoretic mobilities of the γ_c-globulin in the Vt-filtrate and the γ_c-globulin in the untreated colostrum are the same.

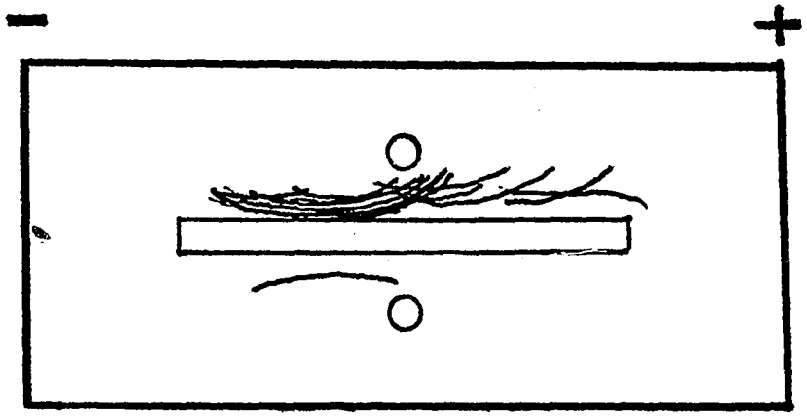

Figure 9: Immunoelectrophoretic patterns of colostral whey (upper well) and Vt-filtrate (bottom well) developed with a rabbit anti-bovine CSF serum.

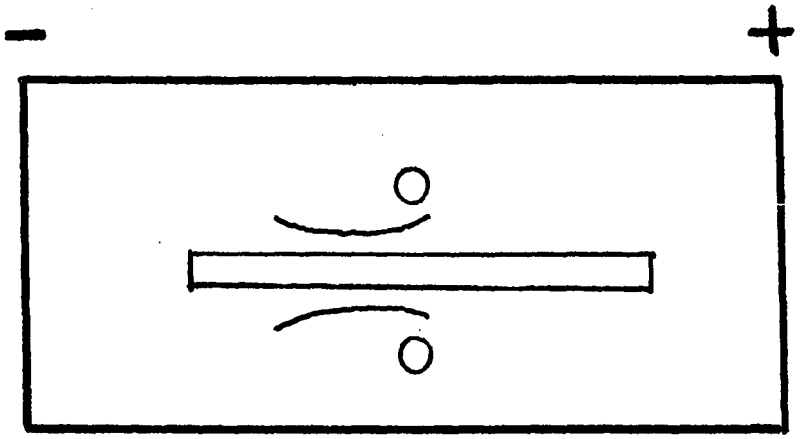

Figure 10: Immunoelectrophoretic patterns of colostral whey (upper well) and Vt- filtrate (bottom well) developed with a rabbit anti-γ_c-globulin serum.

The Vt-filtrate was also analyzed by disc gel electrophoresis. The results are shown in Figure 11. The patterns were obtained with 100 and 200 μg of protein respectively. Both disc gel electrophoretic patterns contained four (4) bands two of which may be seen to be quite close to each other. The Vt-filtrate was next subjected to G-75 chromatography to ascertain whether the four (4) components were sufficiently different in size to be separated by this technique.

D. CHROMATOGRAPHY OF COLOSTRAL VT-FILTRATE ON SEPHADEX G-75:

Two (2) ml of concentrated colostral Vt-filtrate containing 2 mg of protein per 1.0 ml were applied to a Sephadex G-75 column as explained in " Methods " and eluted with Elliott's solution. Five 1.0 ml fractions were collected per hour. The elution pattern is shown in Figure 12. The elution profile contained four (4) peaks and the contents of the tubes were pooled as follows: Fraction C, tubes 50 to 79; Fraction D, tubes 80 to 95 ; Fraction E, tubes 96 to 124 ; Fraction F, tubes 125 to 140. In order to ascertain which peak contained the γ_C-globulin, each of the four fractions were concentrated to 0.1 to 0.2 ml and analyzed by the gel diffusion method with an anti-bovine CSF serum and an anti-γ_C-globulin antiserum. The results are shown in Figure 13. It will be seen by inspection of Figure 12 that the γc-globulin was eluted in the first peak, Fraction C.

When Fraction C was analyzed by disc gel electrophoresis only one (1) band was formed as is shown in Figure 14.

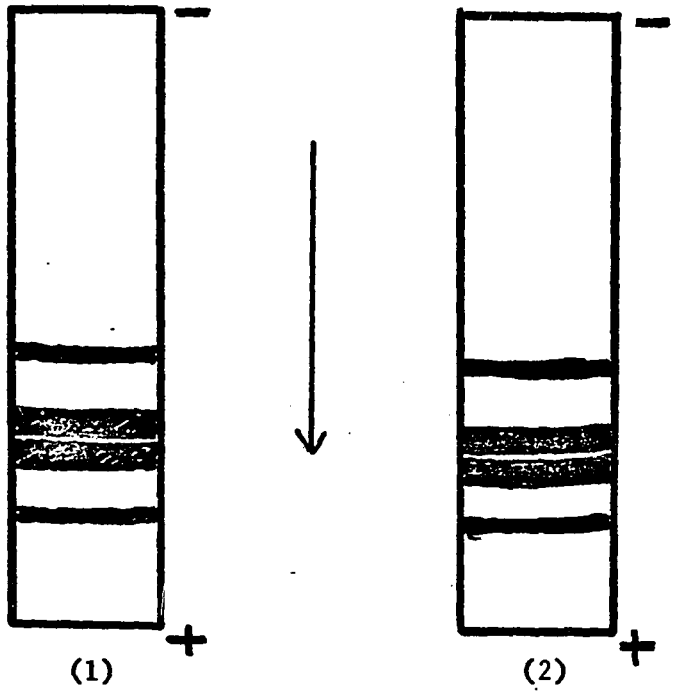

Figure 11: Disc gel electrophoretic pattern obtained with (1) 100 µg of Vt-filtrate protein and (2) 200 µg of Vt-filtrate protein.

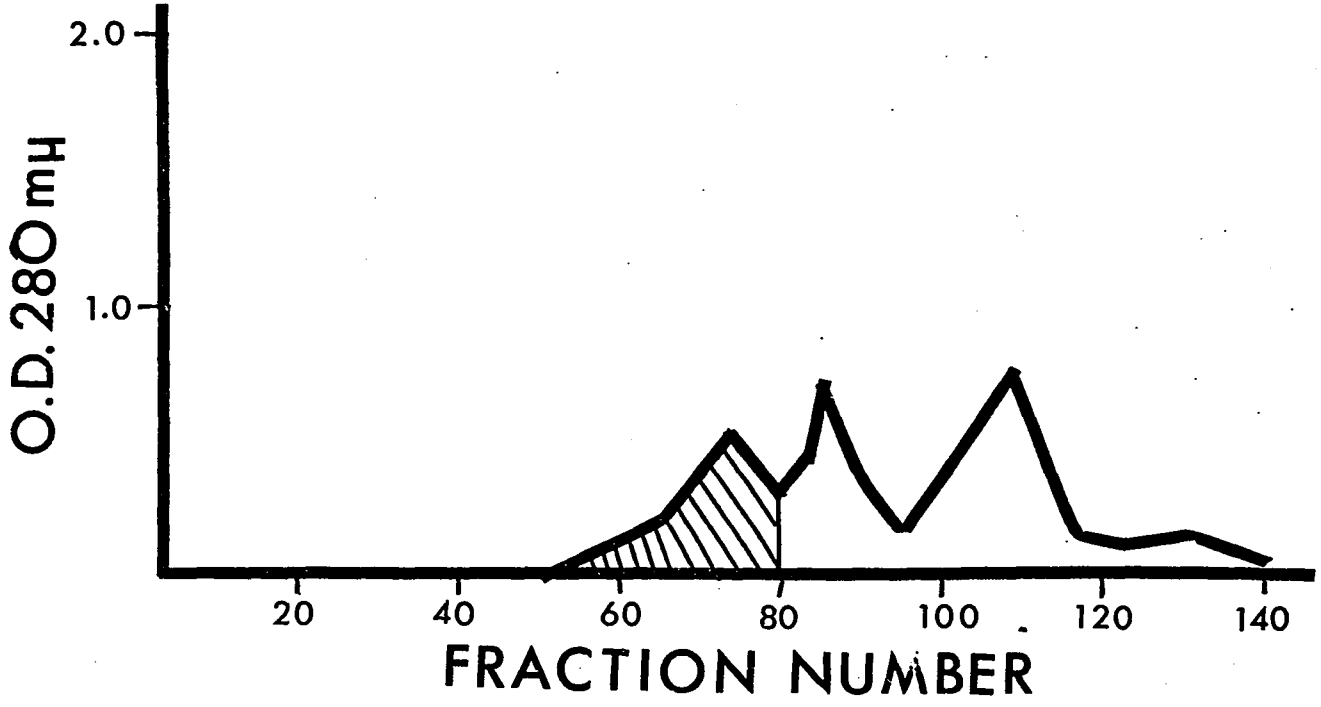

Figure 12. Fractionation of 2 ml of colostral Vt- filtrate (2 mg of protein per ml) on Sephadex G-75.

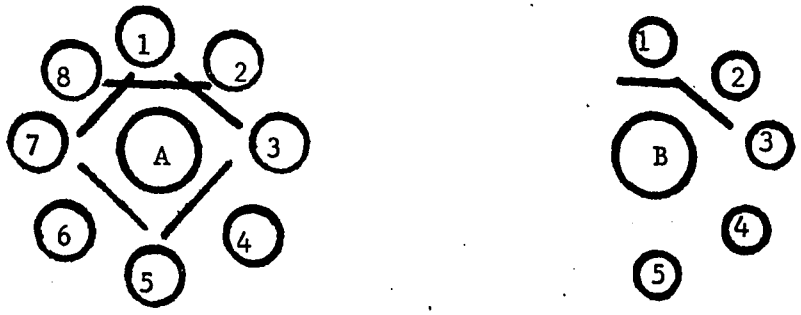

Figure 13: (A). Double diffusion analysis of Fraction C, D, E and F in wells numbered 1, 3, 5 and 7 respectively. Wells numbered 2 and 6 were filled with IgG and wells numbered 4 and 8 were filled with BSA. The center well contained a rabbit anti-bovine CSF serum.

(B). Double diffusion analysis of Fraction C, D, E and F in wells numbered 2, 3 , 4 and 5 respectively and well numbered 1 was filled with a standard bovine CSF (20 mg/ml). The center well contained a rabbit anti-γ_c-globulin serum.

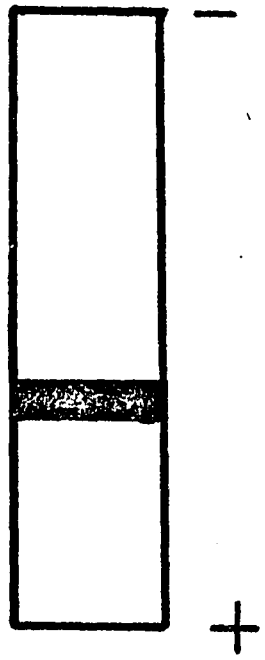

Figure 14: Disc gel electrophoretic pattern obtained with Fraction C, 30 µg of protein was used.

The molecular size of the γ_c-globulin in the Vi-filtrate was determined on a Sephadex G-75 column, that had been previously calibrated with proteins of known molecular weight. The elution volumes are shown in Table 2. It was found that the elution volume (Figure 15) of the γ_c-globulin was 72 ml, which corresponded to a molecular weight of approximately 15,000.

TABLE 2

ELUTION VOLUMES OF VARIOUS PROTEINS FROM A SEPHADEX G-75 COLUMN

Protein	Molecular wt	Ve(ml)
Fraction C from colostral Vt-filtrate	15,000	72
Cytochrome c	12,400	76
Chymotrypsinogen	25,000	61
Bovine serum albumin	67,000	44
Bovine γ-globulin	160,000	40
Blue Dextran	2,000,000	38

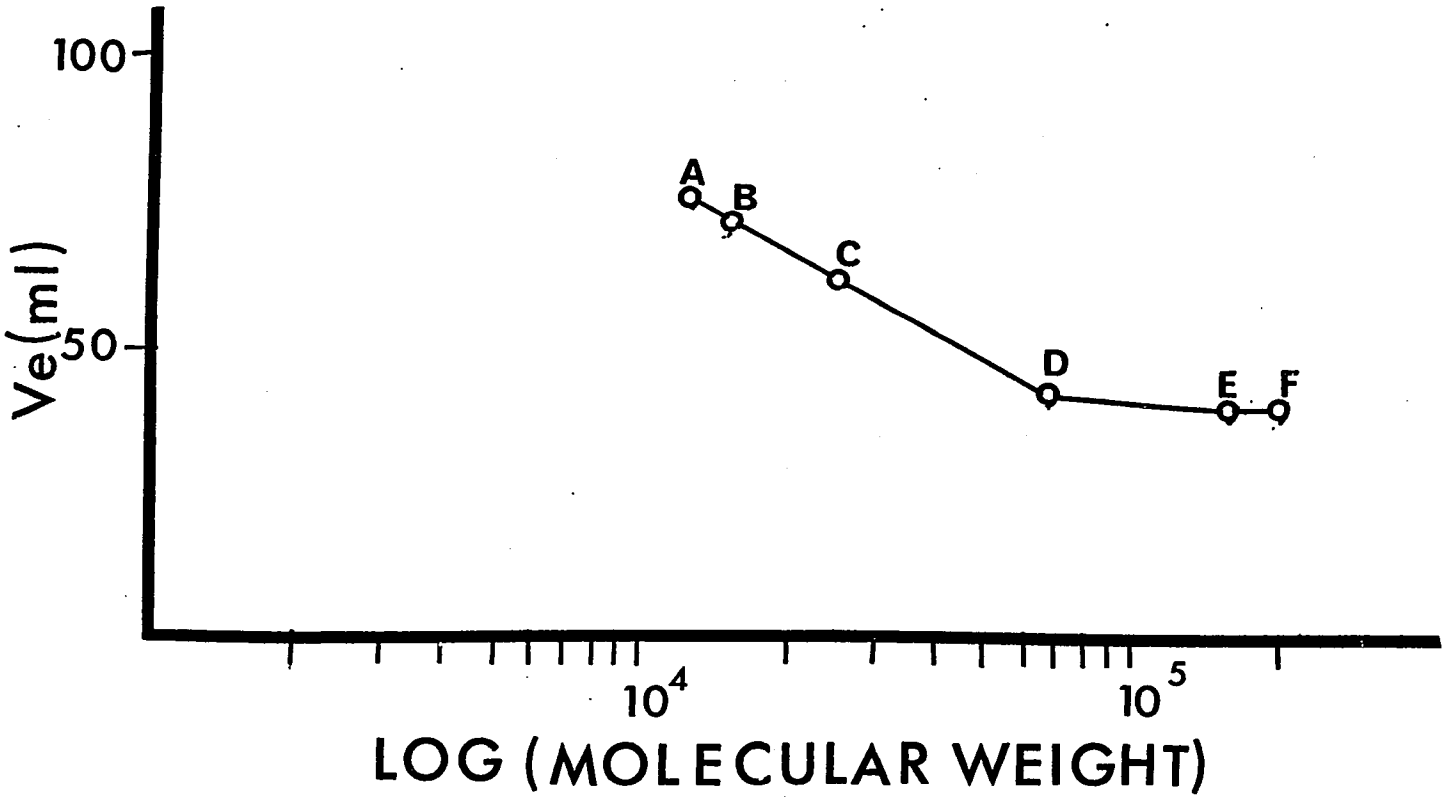

Figure 15. Plot of the elution volume (Ve) against log (molecular weight) for proteins on a Sephadex G-75 column.

A, Cytochrome c ; B, Fraction C from colostral Vt- filtrate ; C, Chymotrypsinogen ; D, Bovine serum albumin ; E, Bovine γ-globulin ; F, Blue Dextran.

E. FRACTION AB OBTAINED FROM CHROMATOGRAPHY ON SEPHADEX G-75:

The γ_c-globulin activity was also found in the concentrated colostral whey after it had been reduced to a suitable small volume in the Visking dialysis tubing. The γ_c-globulin of Fraction AB retained in the concentrate of peak material was partially purified by chromatographying 10 ml containing 100 mg total protein on a 4 x 50 cm Sephadex G-75 column (see methods) using Elliott's solution as the eluting buffer. The elution profile is shown in Figure 16. By inspecting the elution profile and the drawings of the double diffusion analyses (Figure 17A and Figure 17 B), it will be seen that the eluates containing the γ_c-globulin (figure 17A) are contaminated with IgG and contain γ_c-globulin(Figure 17B).

In an attempt to remove the IgG, the fractions (Sephadex G-75 chromatography from above) containing the γ_c-globulin were pooled to form Fraction G and concentrated by pressure dialysis. Two ml of Fraction G containing 4-5 mg total protein were applied to a Sephadex G-100 (2 x 80 cm) and eluted with Elliott's solution. The chromatogram is shown in Figure 18. Disc gel electrophoresis (Figure 19) and double diffusion analyses still showed traces of IgG were still present in the fractions (Fraction H) containing the γ_c-globulin recovered from this procedure.

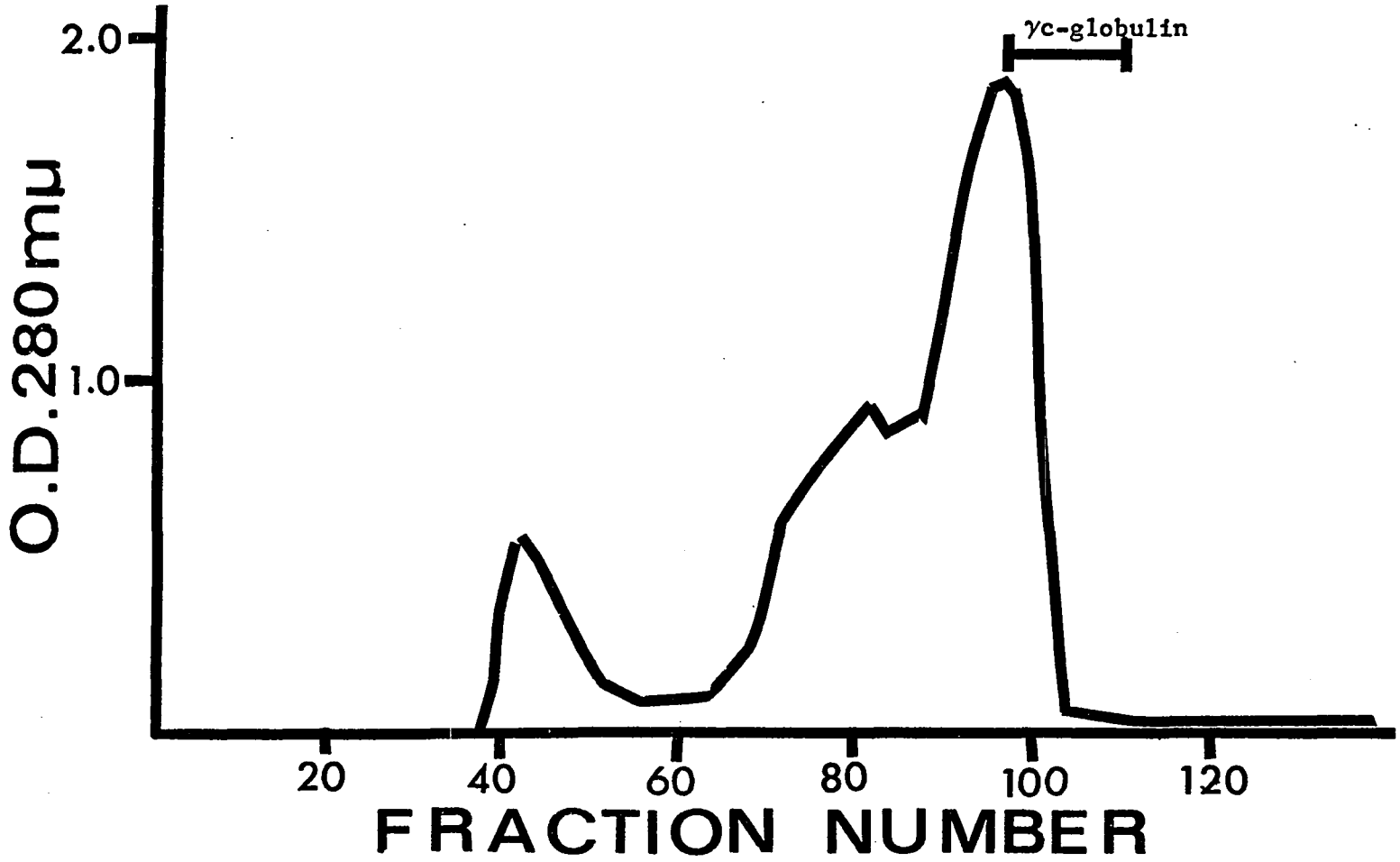

Figure 16. Chromatography of 10 ml of peak material Fraction AB (colostrum) on Sephadex G-75.

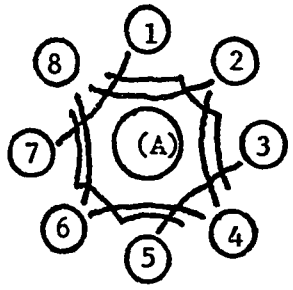

Figure 17 (A): Double diffusion analysis of fractions (wells numbered 1,3,5 and 7) containing the γ_c-globulin after fractionation of Fraction AB on Sephadex G-75. Wells numbered 2 and 6 were filled with IgG and wells numbered 4 and 8 were filled with BSA. The center well contains a rabbit anti-bovine CSF serum.

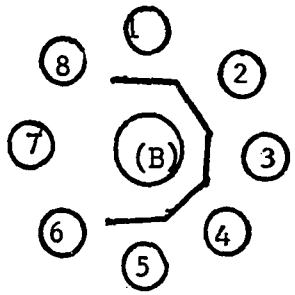

Figure 17 (B): Double diffusion analysis of fractions (wells numbered 2,3,4 and 5) containing the γ_c-globulin after fractionation of Fraction AB on Sephadex G-75. Well numbered 1 was filled with a standard CSF (20 mg/ml). The center well contained a rabbit anti-bovine γ_c globulin serum

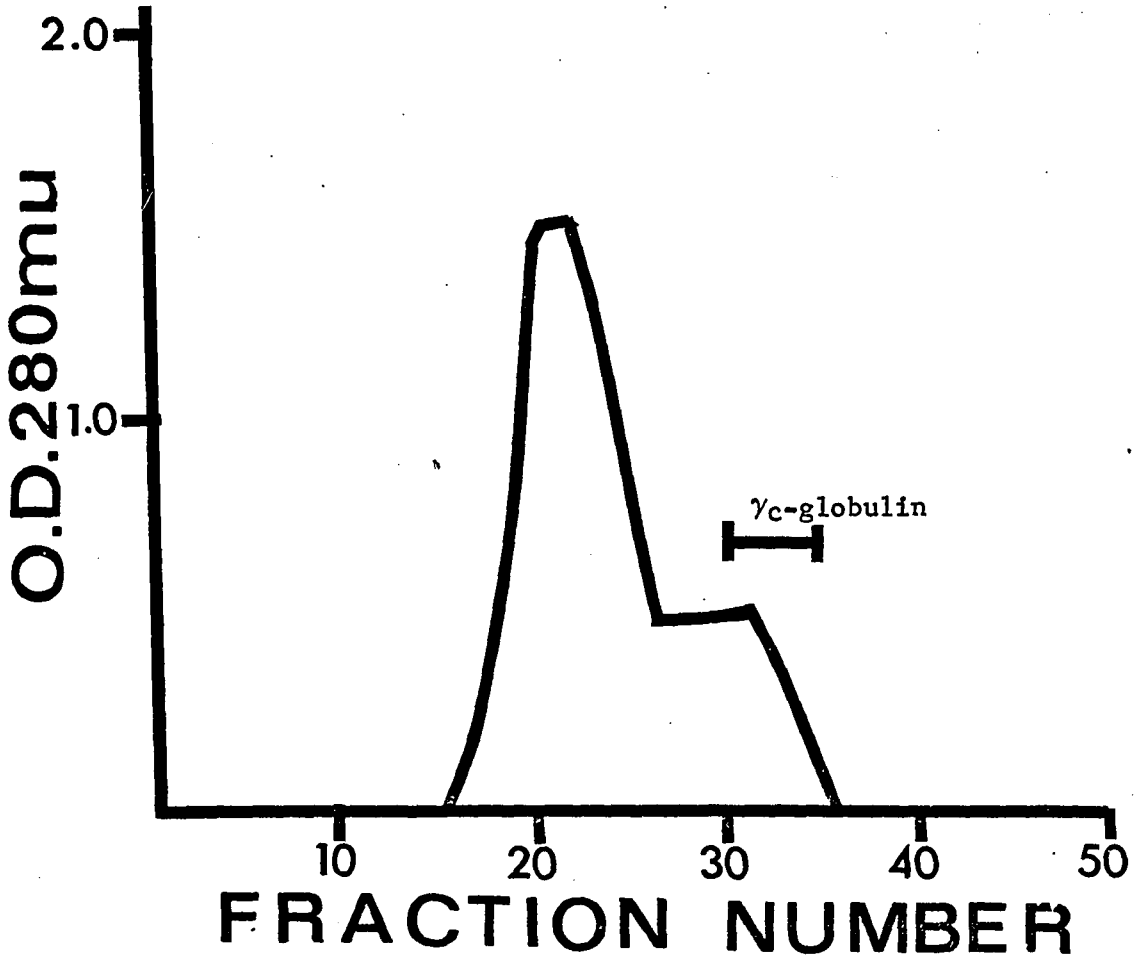

Figure 18: Chromatograpy of 2 ml of Fraction G (4-5mg total protein) on Sephadex G-100.

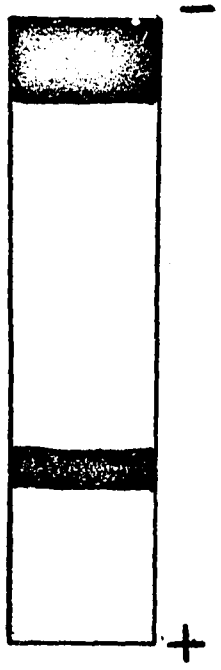

Figure 19: Disc gel electrophoretic profile of Fraction H− from chromatograhy on Sephadex G-100.

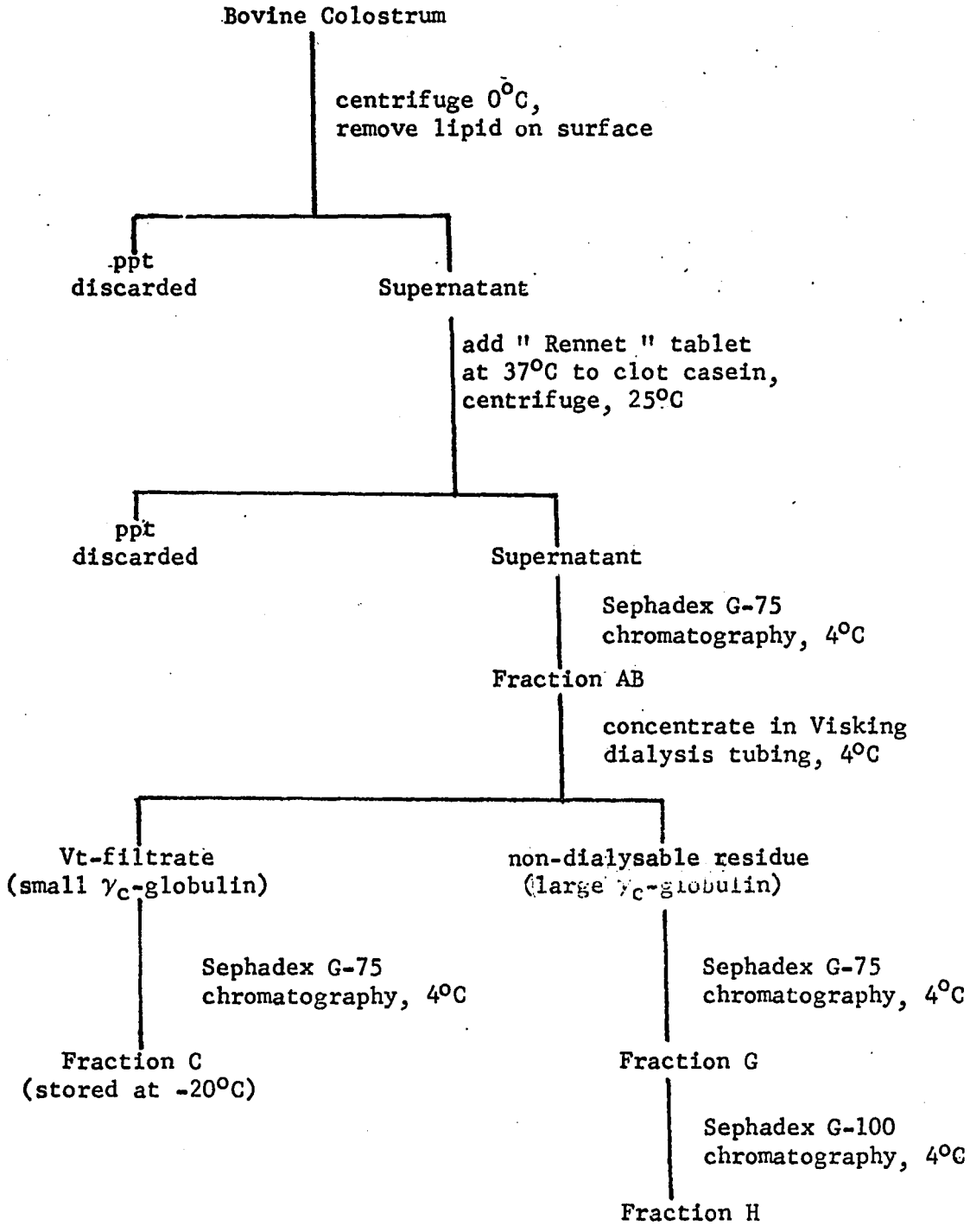

Diagram B: Flow diagram of the isolation of the γ_c-globulin from bovine colostrum

F. DIALYZABLE γ_c-GLOBULIN FROM BOVINE CSF:

When bovine CSF was concentrated in Visking dialysis tubing under slight positive pressure, the concentrated filtrates were examined by double diffusion analyses and found to contain the γ_c-globulin also. The results are shown in Figure 20. The precipitin arcs of the bovine CSF Vt-filtrates do not fuse with the precipitin bands of the standard bovine IgG in wells numbered 2 and 6 or with the precipitin bands formed by the standard BSA in wells numbered 4 and 8, indicating that the bovine CSF Vt-filtrates contained γ_c-globulin but were free from IgG and BSA.

Each concentrate of bovine CSF Vt-filtrate formed one (1) band with a rabbit anti-bovine γ_c-globulin serum and all of them fused with one another as is shown in Figure 21. The fusing of the precipitin bands formed by the four (4) bovine CSF Vt-filtrates and the standard bovine CSF indicate that the Vt-filtrates contain γ_c-globulin.

In Figure 22 is an immunoelectrophoretic analysis of bovine CSF Vt-filtrate. It will be seen that the pattern contains one precipitin band indicating that the Vt-filtrate contains only γ_c-globulin and is free from all the other antigenic proteins of CSF. The electrophoretic mobilities of the γ_c-globulin in the standard bovine CSF and the γ_c-globulin in the bovine CSF Vt-filtrate are identical as is shown in Figure 23.

Although bovine CSF Vt-filtrate contained only the γ_c-globulin, analysis of the bovine CSF Vt-filtrate by disc gel electrophoresis (Figure 24) showed that it contained four (4) to five (5) other non-antigenic proteins or peptides as

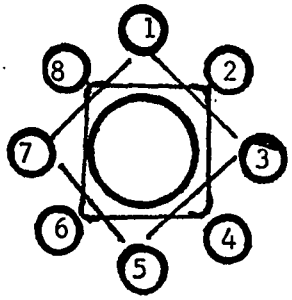

Figure 20: Double diffusion analysis of bovine CSF Vt- filtrates in wells numbered 1,3, 5 and 7 and bovine IgG in wells numbered 2 and 6 and BSA in wells numbered 4 and 8. The center well contains a rabbit anti-bovine CSF serum.

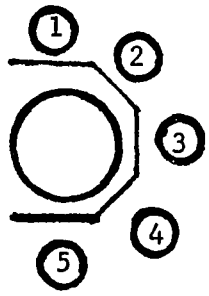

Figure 21: Double diffusion analysis of bovine CSF Vt- filtrates in wells numbered 2,3,4 and 5 and a standard bovine CSF (20 mg/ml) in well numbered 1. The center well contains a rabbit anti-bovine γ_C-globulin serum.

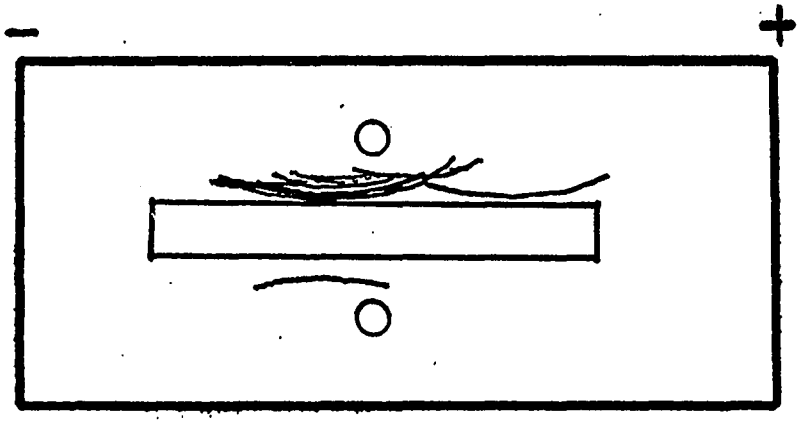

Figure 22: Immunoelectrophoretic patterns of bovine CSF (upper well) and bovine CSF Vt-filtrate (bottom well) developed with a rabbit anti-bovine CSF serum.

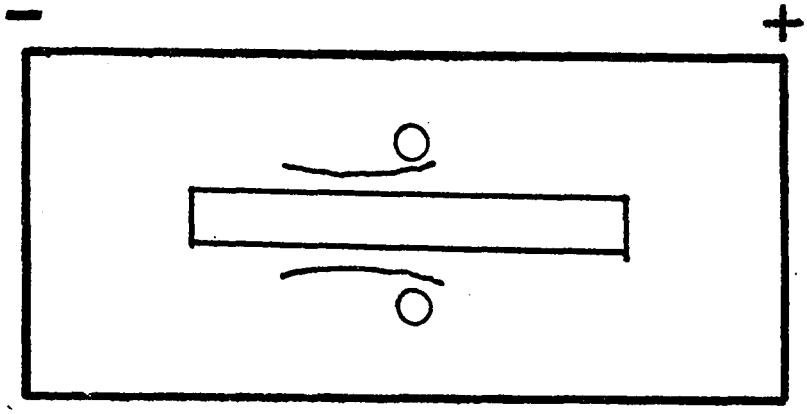

Figure 23: Immunoelectrophoretic patterns of bovine CSF (upper well) and bovine CSF Vt-filtrate (bottom well) developed with a rabbit anti-bovine γ_C-globulin serum.

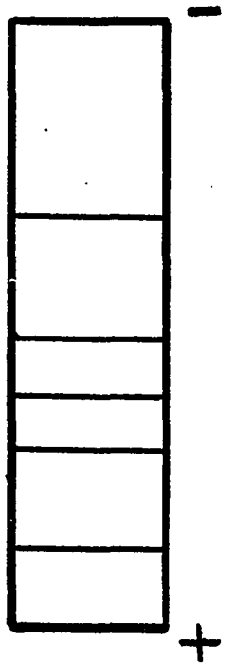

Figure 24: Disc gel electrophoretic pattern of bovine CSF Vt- filtrate.

well. The separation of the components of the bovine CSF Vt-filtrates will be undertaken whenever a sufficient quantity of bovine CSF Vt-filtrate is available.

G. NON-DIALYZABLE γc-GLOBULIN FROM BOVINE CSF:

The γ_c-globulin activity was also found in the CSF retained inside the dialysis tubing after it had been concentrated in the Visking dialysis tubing. The protein was purified by the procedure published by MacPherson and Feldmuller (32) by first removing the albumin by ammonium sulfate precipitation and then subjecting the precipitated globulins to chromatography on Sephadex G-75. The elution profile is shown in Figure 25. The main peak is IgG of CSF. Fractions 45 to 60 contained the γ_c-globulin as determined by double diffusion analyses of which are shown in Figure 26. The protein is free from other antigenic constituents of CSF, but to date, sufficient amounts for disc gel electrophoresis have not been available. This protein has been found to have a molecular weight of approximately 30,000 by gel filtration.

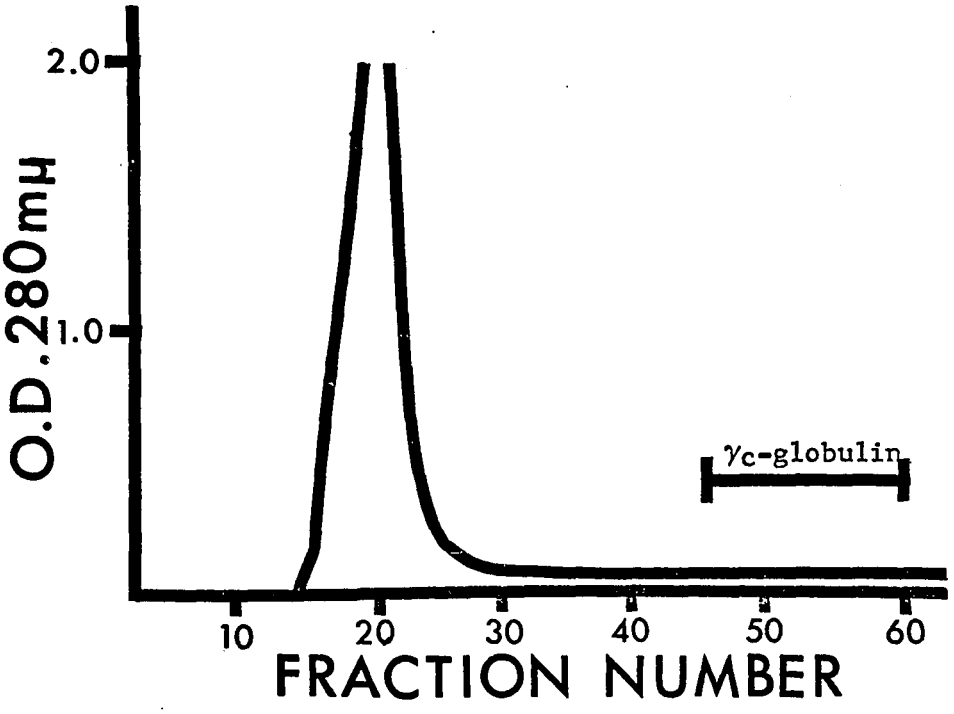

Figure 25: Gel filtration of 10 ml of the globulin fraction (40 mg total protein) of bovine CSF on Sephadex G-75.

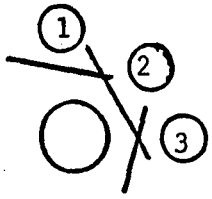

Figure 26 : Double diffusion analysis of Fractions 45 to 60 from chromatography on Sephadex G-75 in well numbered 2 and bovine IgG in well numbered 1 and BSA in well numbered 3. The center well contained a rabbit anti-bovine CSF serum.

H. DIALYZABLE γ_c-GLOBULIN FROM HUMAN CSF:

When human CSF was concentrated in Visking dialysis tubing under pressure the human γ_c-globulin was detected in the concentrated filtrates by double diffusion analyses. (Figure 27). The precipitin arcs formed by the human CSF Vt-filtrates do not fuse with the arcs formed by the standard human IgG in wells numbered 3 and 5 or with the precipitin band formed by the standard human serum albumin in well numbered 1, indicating that the human CSF Vt-filtrates contained γ_c-globulin, but were free from human IgG and human serum albumin.

Each concentrate of human Vt-filtrate formed one (1) band with a rabbit anti-human γ_c-globulin serum and all of them fused with one another as is shown in Figure 28. The fusing of the precipitin bands formed by the three (3) Vt-filtrates and a standard human CSF indicate that the Vt-filtrates contain γc-globulin.

In Figure 29 and 30 are immunoelectrophoretic analyses of human Vt-filtrates. It will be seen that the patterns contain only one precipitin arc, that of the γ_c-globulin, indicating that the Vt-filtrate contains no other antigenic proteins of CSF ; the electrophoretic mobilities of the γ_c-globulin in the standard human CSF and the γ_c-globulin in the Vt-filtrate are the same (Figure 30).

In order to see if this dialyzable form of human γ_c-globulin could be completely removed from the CSF, 80 ml of concentrated CSF was diluted to 160 ml with Elliott's solution and the whole

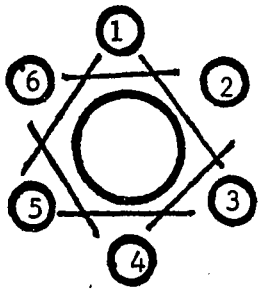

Figure 27: Double diffusion analysis of human CSF Vt- filtrates in wells numbered 2, 4 and 6 and human IgG in wells numbered 3 and 5 and human serum albumin in well numbered 1. The center well contained a rabbit anti-human CSF serum.

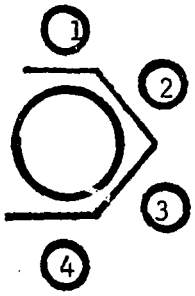

Figure 28: Double diffusion analysis of human CSF Vt- filtrates in wells numbered 2,3 and 4 and a standard human CSF in well numbered 1. The center well contained a rabbit anti-γ_c-globulin serum.

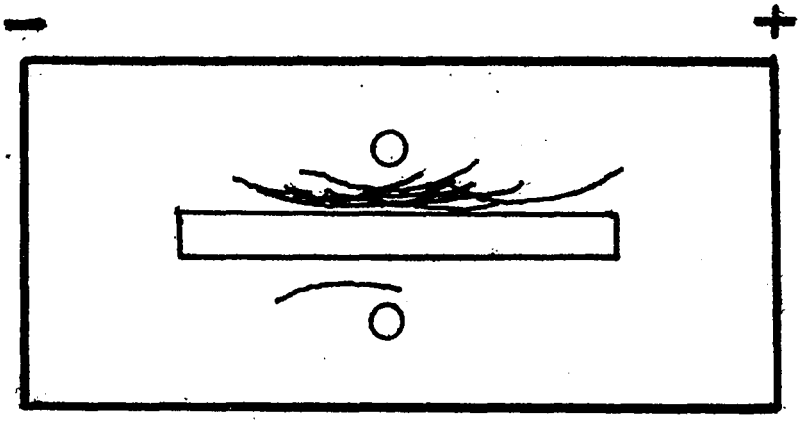

Figure 29: Immunoelectrophoretic patterns of human CSF (20 mg/ml) (upper well) and human CSF Vt- filtrate (bottom well) developed with a rabbit anti-human CSF serum.

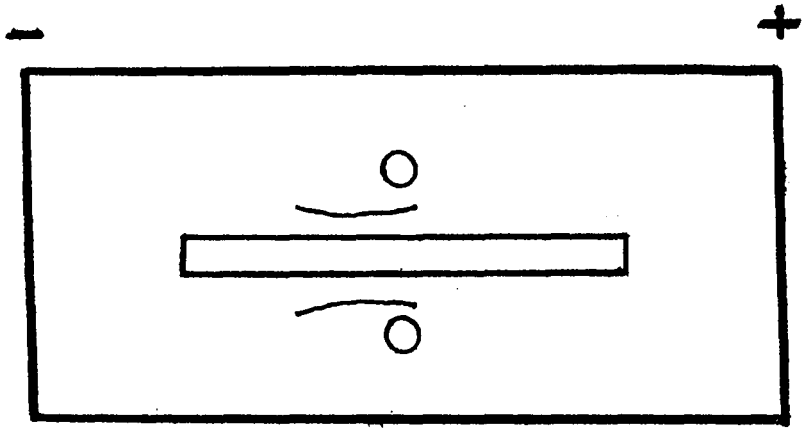

Figure 30: Immunoelectrophoretic patterns of human CSF (20 mg/ml) (upper well) and human CSF Vt- filtrate (bottom well) developed with a rabbit anti-human γ_c-globulin serum.

reconcentrated to the original volume in Visking dialysis tubing under pressure. The procedure was repeated several times with the results shown in Table 3. Each " wash " was examined by gel diffusion analyses using anti-human CSF serum and anti-human γ_c-globulin antiserum. It will be seen that three (3) " washes " were sufficient to remove the dialyzable γ_c-globulin from the CSF. In this experiment the Visking dialysis tubing was suspended in the aspirator bottle so that it was never in contact with the Vt-filtrate, and the γ_c-globulin could not recross the dialysis membrane to enter the CSF again.

TABLE 3

Wash	γ_c-globulin activity	γ_c-globulin activity inside dialysis tubing
1	+++*	+
2	++	+
3	+	+
4	-	+
5	-	+

Although human Vt-filtrate was " immunologically pure ", analysis of the human Vt-filtrate by disc gel electrophoresis (Figure 31) showed that there were at least 4-5 other non-antigenic proteins or peptides present along with the γ_c-globulin. The separation of the components of the human CSF Vt-filtrates will be undertaken as soon as sufficient quantities of human CSF have been accumulated.

* +++ very strong activity ++ strong activity + average activity - negative activity

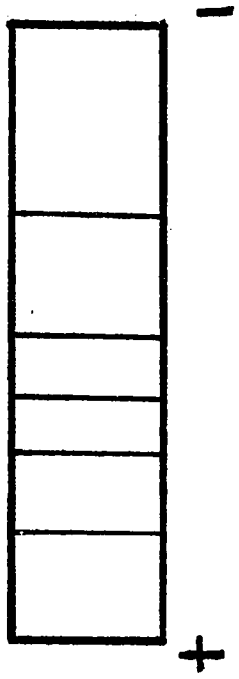

Figure 31: Disc gel electrophoretic pattern of human CSF Vt- filtrate.

I. NON-DIALYZABLE γ_c-GLOBULIN FROM HUMAN CSF:

The γ_c-globulin activity was found in the concentrated CSF after it had been concentrated in the Visking dialysis tubing and could not be removed by " washing ". The non-dialyzable γ_c-globulin was purified in the usual manner (see methods) by first removing the albumin by ammonium sulfate precipitation and then subjecting the precipitated globulins to chromatography on Sephadex G-75. The elution pattern is shown in Figure 32. It can be seen from the elution profile that the non-dialyzable γ_c-globulin was eluted in two (2) different parts of the chromatogram suggesting that the non-dialyzable occurs in two (2) sizes. As usual, the eluates containing the γ_c-globulins did not absorb as a wavelength of 280 mμ as the concentration of the γ_c-globulin is too low to be detected by spectrophotometric analysis. The protein is free from other antigenic constituents of CSF, but to date, a sufficient amount has not been accumulated to perform an analysis by disc gel electrophoresis.

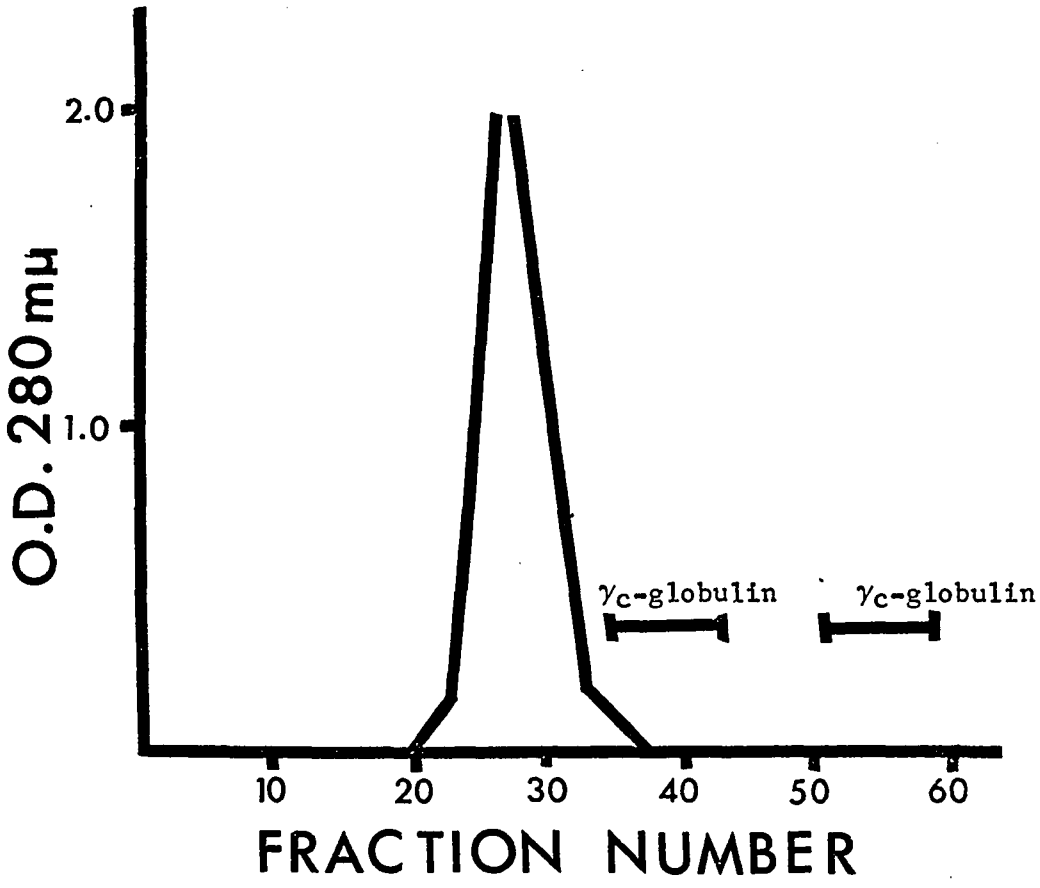

Figure 32: Gel filtration of 10 ml of the globulin fraction (35 mg total protein) of human CSF on Sephadex G-75.

V. DISCUSSION:

When it was discovered that the γ_c-globulin existed in two molecular sizes, it was considered of some importance to obtain sufficient amounts of the two forms so that the biological, chemical and physical properties of the proteins could be determined.

Colostral whey was prepared by removing the fat and casein from colostrum. It was noted in earlier studies (32) that the removal of casein by the iso-electric precipitation at pH 4.0 had a detrimental effect on the immunochemical properties of the γ_c-globulin. In this study, it was found that the casein could be clotted with " Rennet " tablets, a commercial form of rennin, with virtually no loss of γ_c-globulin activity. The use of crystalline rennin caused a 20% loss in γ_c-globulin while iso-electric precipitation with acetic acid resulted in a 40% loss. All the γ_c-globulin preparations studied in the present work were obtained from whey prepared with the " Rennet " tablets.

Two experiments designed to see if the two forms of the γ_c-globulin were present in colostrum before it was treated with rennin or diluted by gel filtration chromatography showed that the small γ_c-globulin was present in the untreated colostrum and could be forced through Visking dialysis tubing under slight pressure along with water, electrolytes and small proteins. The fluid extracted in this manner was called Visking tubing filtrate

(Vt-filtrate). When the same method of concentration was applied to human CSF and bovine CSF, the smaller form of the γ_c-globulin was found in the Vt-filtrate also.

Almost 90% of the protein in colostral whey obtained shortly after parturition (33, 34) is IgG. The colostral whey was first subjected to preparative chromatography on Sephadex G-75 to separate most of the IgG from the γ_c-globulin. The fractions containing the γ_c-globulin were combined and concentrated in Visking dialysis tubing when the smaller form of the protein passed through the dialysis tubing into the filtrate. The Vt-filtrates that had been concentrated partly by rotary evaporation had increased concentrations of electrolytes and often very low concentrations of γ_c-globulin. Earlier, MacPherson and Feldmuller (32) found that the γ_c-globulin was partly destroyed after it had been subjected to reagents such as 60% ammonium sulfate. Thus Vt-filtrates were preferably concentrated by ultrafiltration to avoid the umwanted increase electrolyte concentration.

All Vt-filtrates (from bovine CSF, bovine colostrum and human CSF) formed only one precipitin band when allowed to diffuse against the appropriate rabbit anti-CSF sera and one precipitin band when allowed to diffuse against a rabbit anti-γ_c-globulin sera in Ouchterlony type analyses. The same results were obtained when immunoelectrophoresis was carried out with

rabbit anti-CSF sera. Thus each Vt-filtrate contained only one (1) antigen.

However all the Vt-filtrates were found to contain 4 to 5 other small proteins when examined by disc gel electrophoresis. Fortunately, these small proteins were of different sizes and were eluted at different volumes when the colostral Vt-filtrates were fractionated by chromatography on Sephadex G-75. The γ_c-globulin was eluted in the first. This eluate was concentrated and formed one band when analyzed by disc gel electrophoresis. The molecular weight of the γ_c-globulin recovered from the colostral Vt-filtrate was found to be approximately 15,000 by gel filtration. The bovine and human γ_c-globulin in the Vt-filtrate will be purified by gel filtration chromatography, when sufficient amounts have been collected.

Earlier work by MacPherson and Feldmuller (32) on the γ_c-globulin of CSF was done entirely on the larger molecular form of the γ_c-globulin. The serum albumin was removed from the concentrated CSF by ammonium sulfate precipitation. Then, the CSF globulins were separated by chromatography on Sephadex G-75 to obtain the γ_c-globulin of molecular size of 30,000. In bovine CSF and bovine colostrum the larger γ_c-globulin was eluted in one area of the chromatogram. In contrast, the larger γ_c-globulin of human CSF was eluted in two areas of the chromatogram, indicating that the retained human γ_c-globulin is present in two sizes.

The results obtained from chromatography of colostral whey on Sephadex G-100 suggest that colostrum contains species of IgG having molecular sizes smaller than the conventional 160,000. Thus the Sephadex G-75 chromatogram of CSF globulins contains one main peak while the chromatogram of the globulins of colostrum has two (2) to sometimes three (3) peaks. The γc-globulin can be freed from the contaminating IgG almost completely by chromatography on Sephadex G-100. For complete removal of this IgG, Sephadex G-150 or Sephadex G-200 is needed.

The fact that the γ_c-globulin exists in secretions of the normal brain in two (2) molecular sizes, one of which is one half the size of the other is an interesting phenomenon. The proportions in which the two (2) forms exist has not yet been worked out, but the concentrations of the two forms appear to vary independently. Thus, the " smaller " molecular size can be completely " washed out " of CSF or colostrum without diminishing the amount of the larger molecule.

Studies are being continued to ascertain how the smaller molecule is formed in vivo from the larger form and if the smaller form exists in " organized " tissue. There is evidence that in bovine spinal cord , the γ_c-globulin is not present in the 15,000 molecular size.

V1. SUMMARY:

A method has been described for the isolation and purification of the γ_c-globulins from CSF and colostrum. The steps were as follows:

Colostral whey was fractionated by gel filtration on Sephadex G-75 and the eluates containing the γ_c-globulin were concentrated in Visking dialysis tubing. The Vt-filtrates were concentrated by ultrafiltration and fractionated on Sephadex G-75 to obtain the purified smaller γ_c-globulin. The γ_c-globulin prepared in this manner formed one band on disc gel electrophoresis and had a molecular weight of approximately 15,000 by gel filtration.

Bovine CSF or human CSF was concentrated in Visking dialysis tubing and the Vt-filtrates were concentrated by ultrafiltration. The small γ_c-globulin is the only antigen in the filtrates that can be detected with anti-CSF sera. Disc gel electrophoresis revealed that the Vt-filtrates contained three or four other components.

The larger form of the γ_c-globulin was isolated from the CSF retained in the Visking dialysis tubing by ammonium sulfate precipitation and chromatography on Sephadex G-75. The protein has been found to have a molecular weight of approximately 30,000 by gel filtration.

The larger γ_c-globulin was isolated from colostrum by fractionation on Sephadex G-75 and then rechromatography on Sephadex G-100.

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