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Abstract

An abnormal protein with similar antigenic properties to Fc fragments of IgG, was found in the serum and urine of an eleventh case of heavy (Hgamma1) chain disease (Yok). This protein was purified with ammonium sulfate precipitation and by column chromatography of DEAE cellulose, CM cellulose and Sephadex G-200. The purity of the protein obtained was 98.5%. It was crystallized easily, forming thin hexagonal plates of various sizes. The chemical compositions and physical properties of the protein including viscosity, partial specific volume, diffusion constant, sedimentation constant, frictional ratio, extinction coefficient and iso-ionic point are reported.

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FURTHER STUDIES ON AN ELEVENTH CASE OF HEAVY ($H\gamma 1$) CHAIN DISEASE — PHYSICO-CHEMICAL STUDIES —

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Abstract: An abnormal protein with similar antigenic properties to Fc fragments of IgG, was found in the serum and urine of an eleventh case of heavy ($H\gamma 1$) chain disease (Yok). This protein was purified with ammonium sulfate precipitation and by column chromatography of DEAE cellulose, CM cellulose and Sephadex G-200. The purity of the protein obtained was 98.5%. It was crystallized easily, forming thin hexagonal plates of various sizes. The chemical compositions and physical properties of the protein including viscosity, partial specific volume, diffusion constant, sedimentation constant, frictional ratio, extinction coefficient and iso-ionic point are reported.

The abnormal proteins found in gamma heavy chain disease have antigenic similarity to Fc fragment produced by papain digestion of IgG (1). In several cases, the proteins seem to bear the genetic (Gm) characteristics of the Fc fragment (2–8). The molecular weights were reported to be 52,000–69,500 daltons (2–5, 9). The sedimentation coefficient were also reported to be 2.8–4.1S (4, 6, 8, 10, 11, 12). Carbohydrate content of the proteins ranged from 10 per cent to 21 per cent (1, 10, 14). Amino acid sequence studies of the protein suggested that they have partial deletion of the Fd fragment of the IgG molecule (15–22).

In our case (Yok) structural studies showed that the N-terminal of the protein begins at positions 219 (serine) and 223 (threonine) of the gamma-1 heavy chain, and that an alanine at position 431 seems to be replaced by glycine (23).

The present studies were initiated to determine physico-chemical properties and chemical composition of the proteins.

METHODS

Paper electrophoresis was performed with Toyo Model SE-2 paper electrophoresis system (24). Immunoelectrophoretic analysis was pefrormed by the method of SCHEIDEGGER (25). Moving boundary electrophoresis was carried out in a Hitachi Model HTD-1 apparatus (26). Analytical polyacrylamide electrophoresis was performed according to MAIZEL (27). The immunoglobulins and their molecular subunits were identified by specific monovalent anti sera obtained commercially. The quantitation of serum protein was performed by using the antibody-in agar radial diffusion method (28).

For ultracentrifugal analysis, a Spinco Model E and a Hitachi UCA-1 instruments were employed at a speed of 51,200 or 60,000 r.p.m. Both moving boundary electrophoretic and ultracentrifugal analyses were performed in buffers of 0.1 ionic strength. To cover a wide pH range, glycine-NaOH, barbital-HCl, phosphate, acetate, formate, glycine-HCl and HCl buffers were used. Sodium chloride made up 80% of ionic strength of these buffers, except when 0.1N HCl was used. The sedimentation constant at an infinite dilution was determined in potassium phosphate buffer, pH 7.2, 0.1 ionic strength, with sodium chloride making up 80% of the ionic strength. Partial specific volume determination as well as viscosity and sedimentation equilibrium analyses were also made in this buffer.

The partial specific volume of the protein was determined at 20°C in a 5-ml pycnometer calibrated with distilled water at that temperature. The viscosity measurements were made at 20°C in an Ostwald viscometer with a flow time of 149 seconds for water. The intrinsic viscosity was determined by the method of SCHACHMAN (29).

Acid-base titrations were carried out over the pH range of 2 to 12 on deionized protein at 20°C. Two milliliters of 2.0 to 2.2% solution of the protein in 0.1M KCl were titrated with 0.1N HCl or 0.1N CO_2-free KOH, with the use of a microburette. The number of milliequivalents of acid or base bound per gram of protein at any pH was calculated from the difference between the equivalents of acid or base added and the equivalents of free hydrogen or hydroxyl ions present at that pH.

The ultraviolet spectrum and extinction coefficient at 278 mμ were determined on the deionized protein. Samples of the protein were deionized by passage through a mixed bed-ion exchange resin; Dowex 1×8, formate form and Dowex 50×8, H^+ form. Dry weight was determined after heating to constant weight at 100°C over P_2O_5 in vacuum.

The serum protein was purified from 70 ml of pooled patient serum. It was precipitated with 1.8 M ammonium sulfate at a concentration of 2 gm per cent. The precipitate formed was dissolved and dialysed against 0.01 M sodium phosphate buffer, pH 8.0. The content in dialysing tube was applied to a DE-52 cellulose column (2.0×90 cm) which had been equilibrated with the same buffer and eluted with a linear concentration gradient of 0 0.15 M NaCl containing the buffer, in 4,000 ml. The peak containing Fc fragment was poold and

dialysed against 0.01 M sodium acetate buffer, pH 5.5. This fraction was applied to a CM-52 cellulose column (2.0×90 cm) which was equilibrated with the buffer. A linear concentration gradient elution of 0-0.25 M NaCl containing a buffer in 4,000 ml was employed after washing the column with the buffer. The peak made by the protein was pooled. This fraction was rechromatographed on a CM-52 cellulose column as before and then lyophilized. The dried protein sample was dissolved in 0.01 M sodium phosphate buffer, pH 8.0, dialyzed against 0.14M NaCl containing the buffer, and diluted to 5gm per cent of protein concentration.

Ten ml of this solution was applied to a Sephadex G-200 column (2.0×90cm) which was equilibrated with 0.14M NaCl containing the buffer. A flow rate of 10ml per hour was maintained for all column chromatographies and fractons of 5, 10, or 15ml were collected. Aliquots were analysed for protein by phenol reaction (30) or optical density at 280mμ by using human albumin as a standard.

For isolation of urinary Fc fragment, 65 liters of urine was concentrated to 1/10 volume by lyophilization. The protein was precipitated with saturation of ammonium sulfate and precipitate formed was dissolved in 2 liters of 0.14M NaCl. This fraction was again precipitated with 1.8M ammonium sulfate and then dialysed against 0.01 M sodium phosphate buffer, pH 8.0 containing 0.14M NaCl. The protein in dialysing tube was subjected to a batchweise treatment with 150gm of DE-52 cellulose. The protein was eluted in 3×1.5 liters of the buffer containing 0.15M NaCl. The eluates were pooled and this step was repeated twice. The proteins obtained by the method was dialysed against 0.01 M sodium phosphate buffer and subjected to a DE-52 cellulose column chromatography, a CM-52 cellulose column chromatography, and a Sephadex G-200 gel filtration successively in the same way as the serum protein.

Total neutral sugars were determined by the anthrone reaction (31) directly and after isolation of neutral sugar fraction (32). For the determination of the individual sugars, the proteins were hydrolysed with 2N sulfuric acid for 4, 5 or 6 hours, and they were isolated as described (32). They were identified and quantitated by paper chromatography (31). Sialic acid was measured by the thiobarbituric acid assay and identified by paper chromatography (31). Total hexosamines were determined after hydrolysis in 4N HCl or 2N H_2SO_4 as described (32). The individual hexosamines were identified by paper chromatography (31) and amino acid analyzer.

For the determination of amino acids, the proteins were hydrolysed in grass-distilled constant boiling HCl under nitrogen at 105° for 28 or 40 hours. Amino acid analyses were performed on a Hitachi KLA-3B and a Yanagimoto LC-5S amino acid analyzers.

RESULTS

Purification of the M-protein: On electrophoresis of pooled serum, at pH 8.6, the M-component peak was found to represent approximately 38% of the total proteins. The protein concentration was 8.3gm per cent. More

than 95% of the M-protein was precipitated by 1.8M ammonium sulfate. The second protein peak on the DE-52 column (Fig. 1A) consisted mostly with the M-protein was pooled, and it was purified further with CM-52 columns (Fig. 1B and 1C) and a Sephadex G-200 column (Fig. 1D). Thirty-six per cent of the M-protein in the original serum was recovered after the final step.

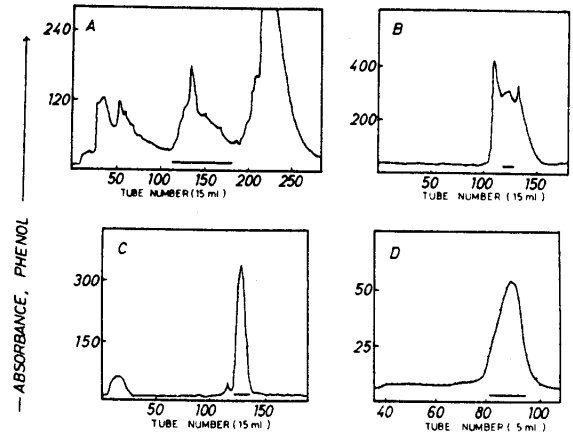

Fig. 1. Elution diagrams of column chromatography
A. DE-52 cellulose column chromatography, B. CM-52 cellulose column chromatography. C. CM-52 Cellulose column rechromatography. D. Sephadex G-200 gel filtration. The areas with bars were pooled and subjected to the next purification step.

The purified serum M-protein fraction formed white fine crystallines when it was dialysed against distilled water. A photomicrograph of such material is shown in Fig. 2A. The crystals were thin hexagonal plates in various sizes ranging 10 to 20 micrometer in the longitudinal dimension. They appeared even in an early purification step, after DE-cellulose column chromatography, by dialysis against distilled water (Fig. 2B). Deionization of the protein resulted in a destruction of the crystalline structure (Fig. 2C). The same results were obtained with the purified urinary M-protein.

For homogeneity studies, the serum M-protein prepared in the above manner was studied by cellulose acetate membrane electrophoresis (Fig. 3), acrylamide gel electrophoresis (Fig. 4), moving boundary electrophoresis (Fig. 5) and in the ultracentrifuge (Fig. 6) over a wide pH range. By these techniques it appeared homogeneous over the entire pH range, from pH 1.8 to 11.2. The boundary electrophoresis was performed at pH 1.8, 2.2, 3.1, 3.6, 4.0, 5.4, 6.3, 7.2, 8.8, 9.8 and 11.2. Several electrophoretic patterns are shown in Fig. 5. The isoelectric point obtained from this experiment was

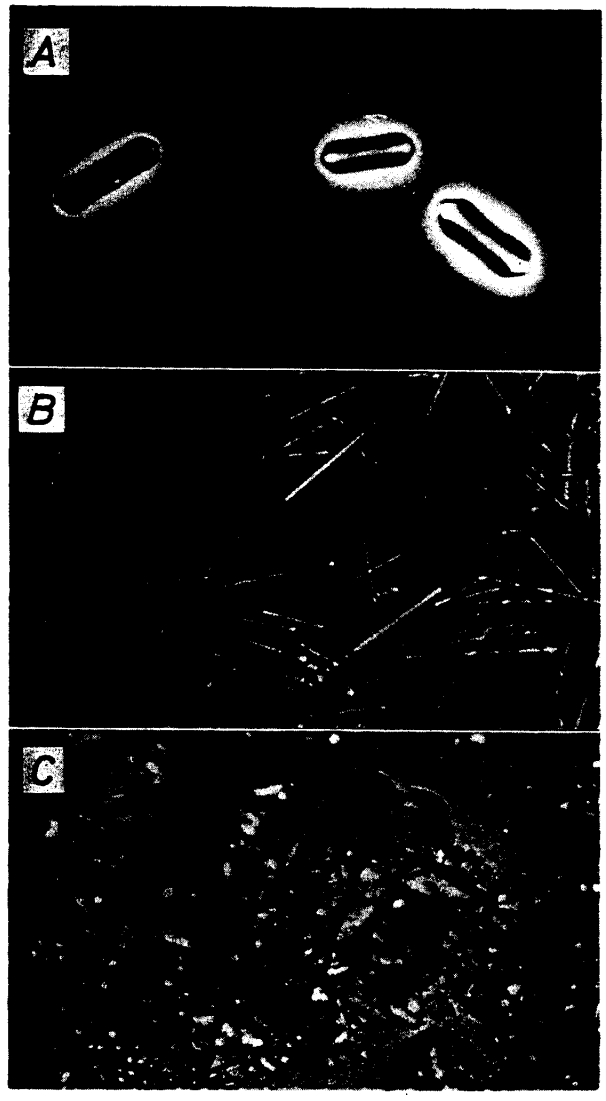

Fig. 2. Photomicrographs of crystalline serum M-component

A. Phase contrast microscopic figure of the purified protein. B. Crystals formed in the early purification step, after DE-52 column chromatography. C. Deionized crystals of the purified protein. Note a hexagonal structure in various sizes ranging 10 to 20 μ in the longitudinal dimension. Original magnification, $\times$500.

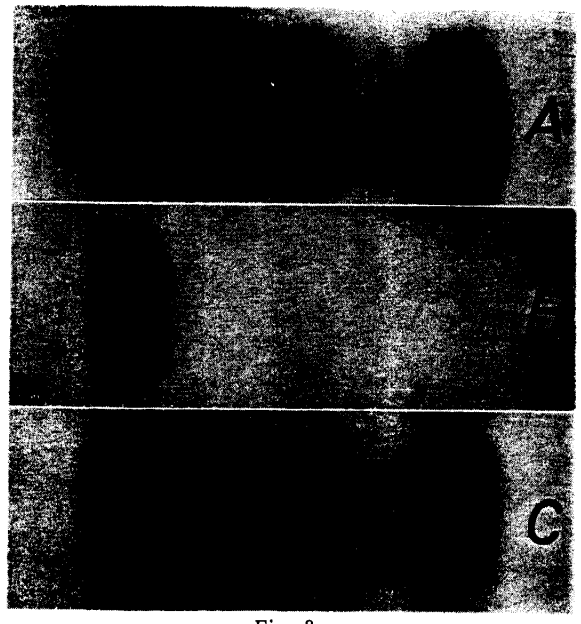

Fig. 3.

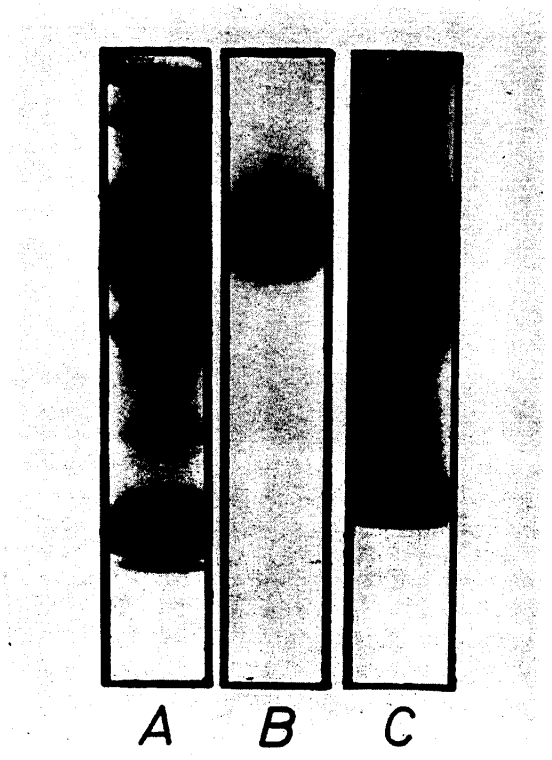

Fig. 4.

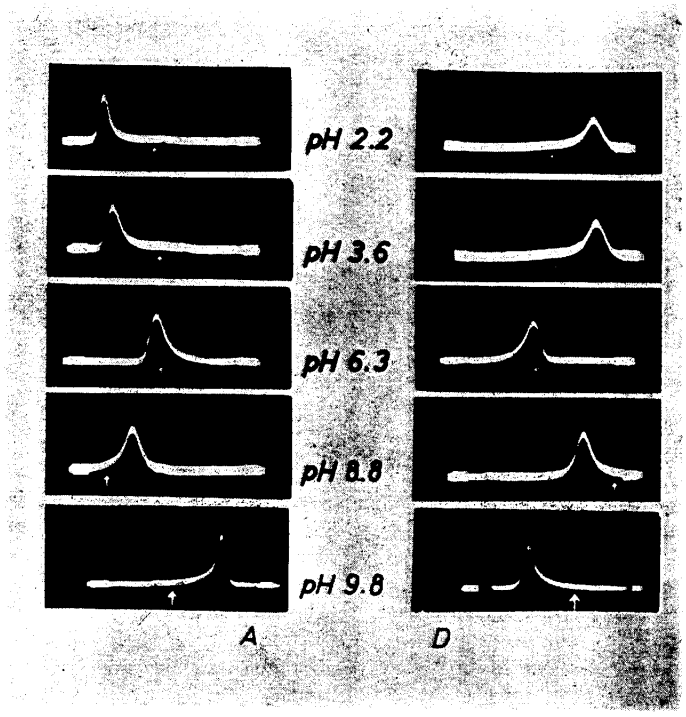

Fig. 5. Ascending (A) and descending (D) electrophoretic patterns of purified serum M-protein at several pH values.

They were run for 60 minutes. The buffers were as follows: pH 2.2, glycine-NaCl; pH 3.6, formate-NaCl; pH 6.3, phosphate-NaCl; pH 9.8, glycine-NaCl. Ionic strength was 0.1, NaCl made up 80% of it. The salt boundaries are showed by arrows. Note symmetric patterns in all pH range.

pH 7.00. The sedimentation studies were carrid out at pH 1.8, 3.2, 4.7, 7.2, 8.6 and 9.7. The patterns at several pH values are shown in Fig. 6. They are symmetric in shape suggesting its molecular homogeneity. Immunoelectrophoretic pattern of the purified serum M-protein was shown in Fig. 7. Only a small arc made by transferrin in addition to a large one precipitated by M-component was observed and transferrin was quantitated as 1.5% of total protein by quantitative single radial immunodiffusion method.

Fig. 3. Electrophoretic patterns on cellulose acetate membrane.

A. Normal human serum. B. Purified M-component. C. Original serum. The anode is at the right.

Fig. 4. High pH discontinuous acrylamide electrophoresis.

A. Original serum. B. Purified serum M-component. C. Urine of the patient. The anode is at the bottom.

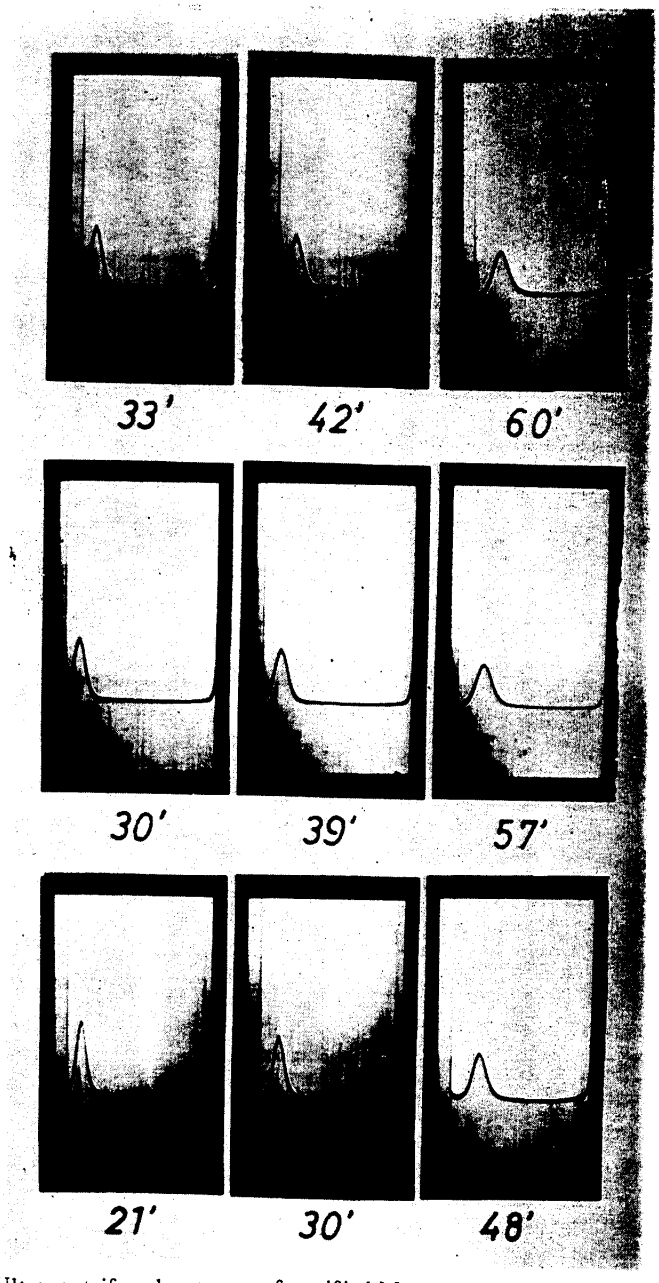

Fig. 6. Ultracentrifugal patterns of purified M-component at several pH values.
The buffers are used as follows: pH 4.7, acetate-NaCl (upper), pH 7.2, phospohate-NaCl (middle), and pH 8.6, Veronal-NaCl (lower). All the buffers were 0.1 ionic strength with NaCl making up 80% of the ionic strength.

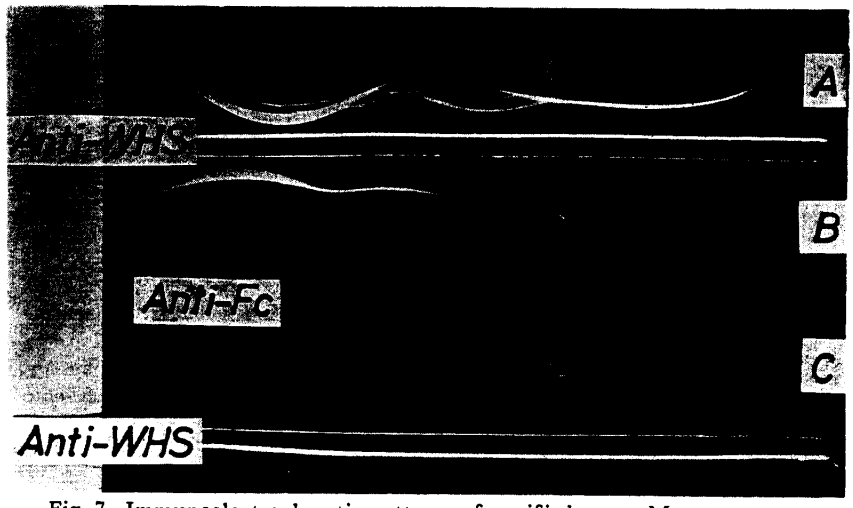

Fig. 7. Immunoelectrophoretic patterns of purified serum M-component.
A. Normal hunan serum, B. Original patient's serum, C. Purified serum M-component. The protein concentration of purified M-component was 5 gm. per cent. Note only a small amount of transferrin is present in the preparation. Anti-WHS: anti whole human serum (rabbit). Anti-Fc: anti Fc fragment (rabbit).

The physico-chemical properties and chemical compositions: The protein gave an ultraviolet spectrum characteristic for a protein, with a maximum at 278 mμ. The extinction coefficient of the deionized protein in 0.1 KCl with 0.01 M sodium phosphate buffer, pH 7.2, corrected to dry weight, was $E^{1\%}_{1cm} = 13.2$. The average partial specific volume at pH 7.2 was $V_{20} = 0.685$ ml per gm. Values were obtained at protein concentrations of 4.68, 3.51, 2.34 and 1.17%. The values of V_{20} at these concentrations were 0.692, 0.679 0.673 and 0.693, respectively.

The molecular weight of the protein was determined by Archibald method to be 59,800. The diffusion constant calculated from the values given for S, M and V_{20} was $D^{\circ}_{20,W} = 4.59 \times 10^{-7} cm^2$ per second. The frictional ratio, f/f_0, calculated from the values of $D^{\circ}_{20,W}$, V_{20} and molecular weight, was 1.83. The axial ratios obtained from frictional ratio by Perrin's ralation, on the basis that the protein is an unhydrated ellipsoid of revolution, are 16.0 for prolate ellipsoid and 21.9 for an oblate ellipsoid.

Viscosity experiments performed in the pH 7.2 buffer are plotted as specific viscosity per unit concentration, $\eta sp/c$, against concentration by the method of least squares in Fig. 8. From the intercept, the value for the intrinsic viscosity at pH 7.2, $[\eta]_{20}$ was found to be 8.4 ml per gm. The values of the viscosity increment, calculated from the intrinsic viscosity, $[\eta]$ and the partial specific volume, V_{20}, from the relation $= 100[\eta]/V_{20}$, was found to be

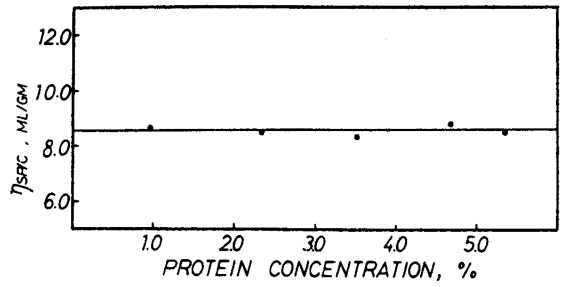

Fig. 8. A plot of specific viscosity per unit concentration against concentration of serum M-component.

The determinations were carried out in a phosphate-sodium chloride buffer, pH 7.2, 0.1 ionic strength, with the sodium chloride made up 80% of the ionic strength.

12.3 at this pH. The axial ratios obtained from the viscosity increment from Shimha's relation on the basis that the molecule is an unhydrated ellipsoid of revolution are 8.7 for a prolate ellipsoid and 16.3 for an oblate ellipsoid. These were of low values comparing to the axial ratios calculated from the frictional ratio. The axial ratios of the urinary M-protein calculated from the frictional ratio and the viscosity increment gave simillar values.

In ultracentrifugal analyses in weak acid and in weak alkaline, the values were lower than those determined at pH 7.2. However, at the pH further apart from the isoelectric point, an increase in the sedimentation rate was observed. At pH 1.8, the sedimentation coefficient $S_{20,w}$ for 1.50% solution was 4.10S; at pH 3.35 for a 0.94% solution, 3.36S; at pH 4.70 for a 0.92% solution, 3.46S; at pH 7.20 for a 0.89% solution, 3.52S; at pH 8.6 for a 0.90% solution, 3.47S; at pH 9.7 for a 1.50% solution was 3.52S. Changes in the molecular shape and interactions between the protein molecule could account for the differences in the sedimentation rate observed at various pH values. Molecular aggregation of the protein might occur at a strong acidic condition and yield a large S value.

To determine whether there was adequate dampening of the primary charge effect at the ionic strength of 0.1 at which these determinations were carried out, the protein was studied in the ultracentrifuge at the same pH but at the higher ionic strengths of 0.46, 0.36, and 0.26, achieved by the presence of a higher concentration of NaCl. The $S_{20,w}$ of a 1.00% protein solution at these higther ionic strengths were 3.45S, 3.55S, and 3.49S, compared to a $S_{20,w}$ or 3.52S at 0.1 ionic strength at this protein concentration. The effect of protein concentration on the sedimentation constant of the purified serum M-component was also examined and no concentration dependency was observed (Fig. 9). The isoionic point determined from the pH of a 2% solution of the

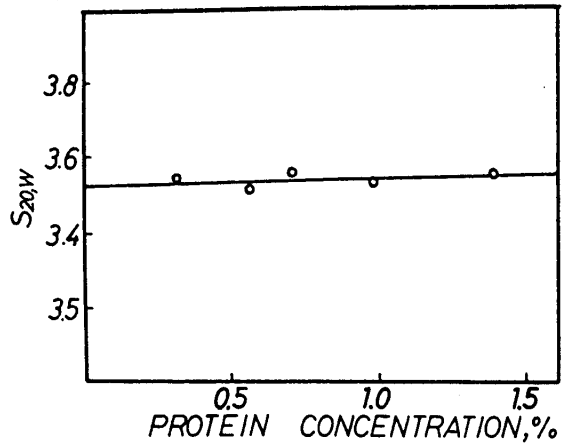

Fig. 9. Effect of protein concentration of the sedimentation constant of serum M-component.

The determinations were made in phosphate-sodium chloride buffer, pH 7.2, ionic strength 0.1, with sodium chloride contributing 80% of the ionic strength.

TABLE 1. AMINO ACID AND CARBOHYDRATE COMPOSITION OF SERUM AND URINE PROTEINS

Amino acids and carbohydrates	serum protein	urinary protein
Lysine	8.33	9.23
Histidine	3.40	3.88
Arginine	3.73	3.17
Aspartic acid	9.71	9.45
Threonine	6.03	6.11
Serine	6.40	6.55
Glutamic acid	12.04	12.40
Proline	7.97	8.41
Glycine	2.79	2.86
Alanine	1.81	1.83
Half cystine	2.66	2.32
Valine	8.70	8.68
Methionine	0.94	0.97
Isoleucine	1.71	1.71
Leucine	7.64	7.93
Threonine	5.98	5.10
Phenylalanine	3.75	3.62
Glucosamine	2.26	2.39
Galactose	1.37	1.29
Mannose	1.83	1.71
Fucose	0.29	0.30
N-acetyl neuraminic acid	0.66	0.52
Total	100.00	100.00

All data are expressed as gm residue per 100 gm protein.

deionized protein in 0.1 M KCl was pH 6.92 (Fig. 10). As shown in Table 1, the protein has 6.5% of carbohydrates in its molecule. The neutral sugars were identified as fucose, mannose and galactose as shown in Fig. 11. The amino sugar was identified as glucosamine by paper chromatography in the Fischer-Nebel solvent system and in an amino acid analyzer. On paper chromatography of the sialic acid fraction, only a single spot corresponding to N-acetylneuraminic acid was identified. The amino acid composition is shown in Table 1.

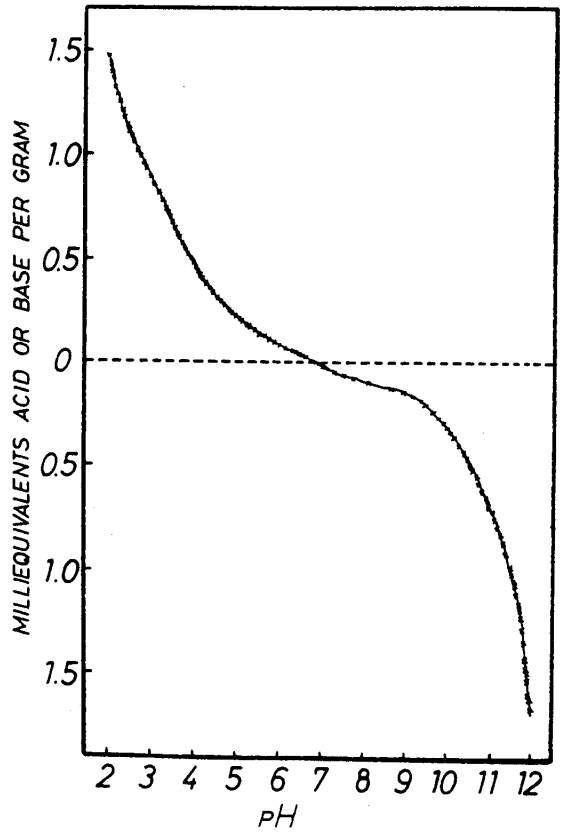

Fig. 10. Titration curve of deionized serum M-component
The milliequivalents of acid or bases added are expressed per gram of dry weight of the protein.

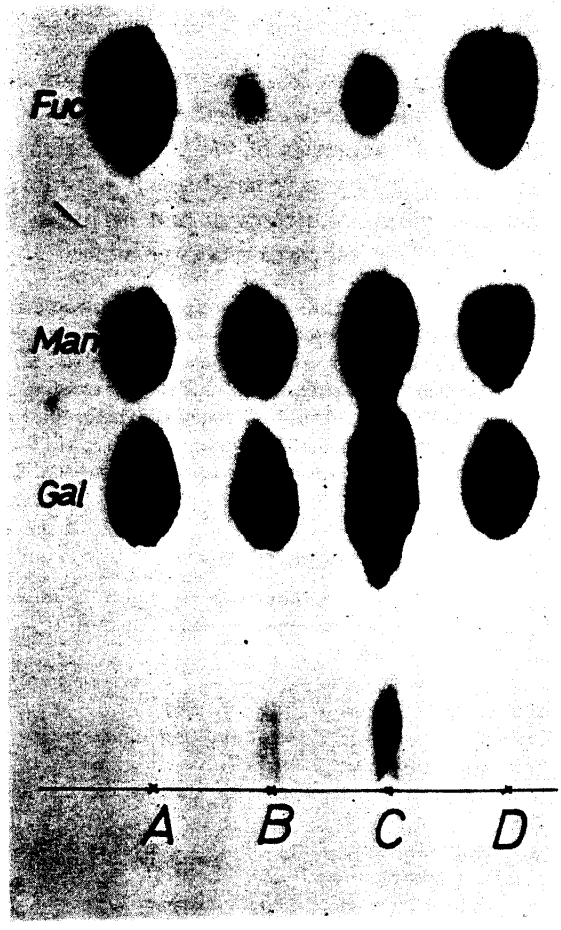

Fig. 11. Paper chromatography of neutral sugar fractions

A and D, standards; B. Serum M-component; C. Urinary M-component. They were developed in the solvent system of n-butanol: ethanol: water = 10 : 1 : 2, and located by silver nitrate. The neutral sugar fraction was prepared by the method described (35). Fuc: fucose, Man: mannose, Gal: galactose.

DISCUSSION

The abnormal protein was purified from patient's serum and urine separately which were 98.5 and 96.5% in terms of purity checked by immunoelectrophoresis and quantitated by single radial immunodiffusion. The analysis of sedimentation gave a lower value than Cra-protein belonging to the same subtype (1). The value obtained by analysis of the whole serum gave a higher S value than that of purified one (8). The proteins purified were easily crystallized by dialysis against distilled water. They were more

uniform than crystallizable fragment of IgG produced by papain digest (33-35).

The carbohydrate content was less than that of Cra-protein while amino acid composision was very similar to each other (13, 34).

Since physicochemical properties of the protein such as isoelectric point, isoionic point, extinction coefficient, partial specific volume, specific viscosity, intrinsic viscosity, viscosity increment and diffusion constant have not been described in the past, no comparison was possible.

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