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John L. Fahey

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Research Article

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HETEROGENEITY OF MYELOMA PROTEINS

By JOHN L. FAHEY

(From the Metabolism Service, National Cancer Institute, Bethesda, Md.)

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The normal gamma globulins are notably heterogeneous in electrophoretic properties, extending in a continuous spectrum throughout a wide portion of the serum globulins in man and many other species (1). In contrast, the serum myeloma proteins found in patients with malignant plasma cell disease form discrete electrophoretic peaks. The apparent homogeneity of human myeloma proteins has been emphasized on the basis of observations made by zone electrophoresis, immunoelectrophoresis, and ultracentrifugation (2–4).

The first evidence that some myeloma proteins might be heterogeneous came from ultracentrifugal analyses which showed that a few myeloma sera contained 9S, 11S, or 13S components, or all, as well as 6.6S components (5–10). Investigation of myeloma sera by means of starch gel electrophoresis also indicated that some myeloma proteins were heterogeneous. Owen, Got, and Silberman (11) studied 16 sera and noted that in seven, 44 per cent, the myeloma proteins were composed of several closely grouped components and that in six, 38 per cent, a less tightly banded pattern of heterogeneity was present. Flynn and Stow (12) reported that three of 11 myeloma proteins contained two to five components, and Fine, Creyssel, and Morel (13, 14) observed heterogeneity in some myeloma proteins of beta mobility but not in any of 15 sera with myeloma proteins of gamma globulin mobility. Laurell (15) reported that 24 per cent of 226 M components were heterogeneous on starch gel electrophoresis. Engle, Woods, Castillo, and Pert (16) found 27 per cent of myeloma proteins had two or more components and noted that in some of these cases there was "periodicity in the placement of peaks while in others the peaks were in widely and irregularly separated regions of the gel." The basis for the heterogeneity, however, was not clear, since the components of each myeloma protein had not been separated and studied in detail.

Askonas (17) detected five or more components on starch gel electrophoresis of the myeloma protein produced by the transplantable mouse plasma cell tumor X5563, and separated the components by diethylaminoethyl (DEAE)-cellulose chromatography. She found that the components had the same ultracentrifugal properties and concluded that the heterogeneity of the X5563 myeloma protein was due to differences in net electrical charge. Recently, 20 different mouse myeloma globulins were studied in detail, and all were found to be heterogeneous (18). Two types of heterogeneity were observed on starch gel electrophoresis. The first type, termed electrophoretic heterogeneity, was revealed by myeloma proteins composed of multiple (three or more), closely approximated, discrete bands, apparently differing in net charge. This was the same pattern described by Askonas (17) and was seen only with γ-myeloma proteins. The second type of heterogeneity was characterized by the presence of several, widely spaced, less discrete myeloma protein components. This latter pattern was observed only with β_{2A}-myeloma proteins which contained 6.6, 9, 11, and 13S components in the ultracentrifuge. It seemed probable that the migration of the larger ultracentrifugal components was impeded by the starch gel, giving rise to the slower migrating bands. On the basis of evidence that the 9, 11, and 13S proteins were made up of polymerized 6.6S components (18), it was suggested that this form of heterogeneity was due to polymer formation rather than electrophoretic differences. Preliminary studies of human myeloma proteins in this laboratory (19) were in accord with the findings of Owen (11), indicating that most myeloma proteins were heterogeneous and that two types of heterogeneity occurred, similar to those seen in the mouse.

The finding that heterogeneity is the common, rather than the uncommon, feature of myeloma proteins has a dual significance. In one case, it points to the question of whether multiple myeloma is a malignancy of one cell line producing multiple proteins or whether multiple tumors (myelomas)

TABLE I

Starch gel electrophoretic patterns in 77 myeloma sera

Immunochemical type	Total number	Electrophoretic heterogeneity	Polymer-type heterogeneity	Single component
γ-Myeloma proteins	58 (75%)	39 (67%)		19 (33%)
β_{2A}-Myeloma proteins	19 (25%)		15 (79%)	4 (21%)
Total	77	54 (70%)		23 (30%)

coexist, each producing a single protein product. In a different context, the limited heterogeneity in a myeloma protein is relevant to the problems in physical chemistry posed by the far more extensive heterogeneity among normal gamma globulins. For these reasons the present studies of the myeloma proteins in 77 sera were undertaken. Components of representative myeloma proteins were separated and characterized in detail.

METHODS

Sera from 77 patients with morphologically proven multiple myeloma were studied by starch gel electrophoresis, immunoelectrophoresis, and paper electrophoresis. Ultracentrifugal analyses were made on 60 sera. In one experiment, sera from C_3H/He mice bearing the SPC-1 plasma cell tumor or the 5647 plasma cell tumors (18, 20) were utilized. Myeloma sera without myeloma proteins and sera having Bence-Jones proteins or macroglobulinemic macroglobulins were not included in the present study.

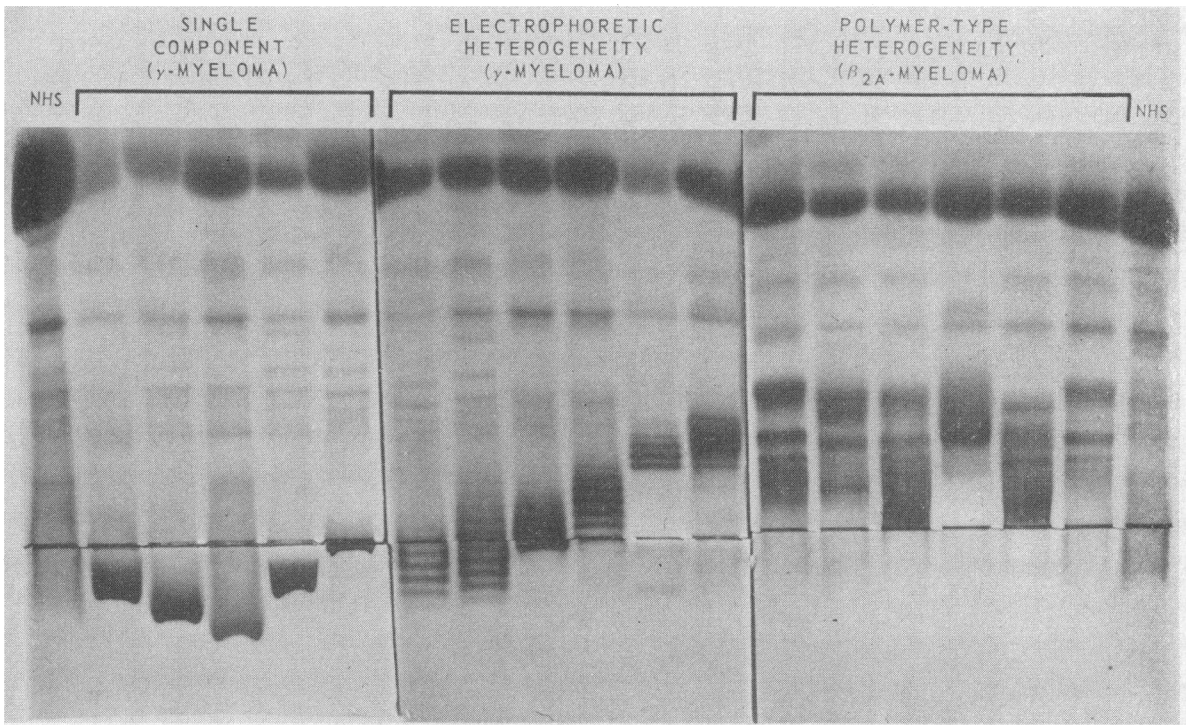

FIG. 1. STARCH GEL ELECTROPHORESIS OF REPRESENTATIVE MYELOMA SERA SHOWING SINGLE COMPONENT MYELOMA PROTEINS (SERA 2–6, COUNTING FROM LEFT), ELECTROPHORETIC HETEROGENEITY OF MYELOMA PROTEINS (SERA 7–12), AND POLYMER-TYPE HETEROGENEITY OF MYELOMA PROTEINS (SERA 13–18). A normal human serum (NHS) is included for reference purpose at each end of the pattern. Starch gel electrophoresis was carried out at room temperature for 5 hours at 390 volts with a discontinuous buffer system having 0.05 M pH 8.8 glycine buffer in the starch gel, 0.1 M pH 9.2 Tris borate buffer in the bridge chamber and ⅛ saturated NaCl in the electrode reservoir. Sliced sections of starch gel were stained with amino black to reveal the protein components. Sera were diluted to obtain myeloma protein concentrations of 5 to 10 mg per ml.

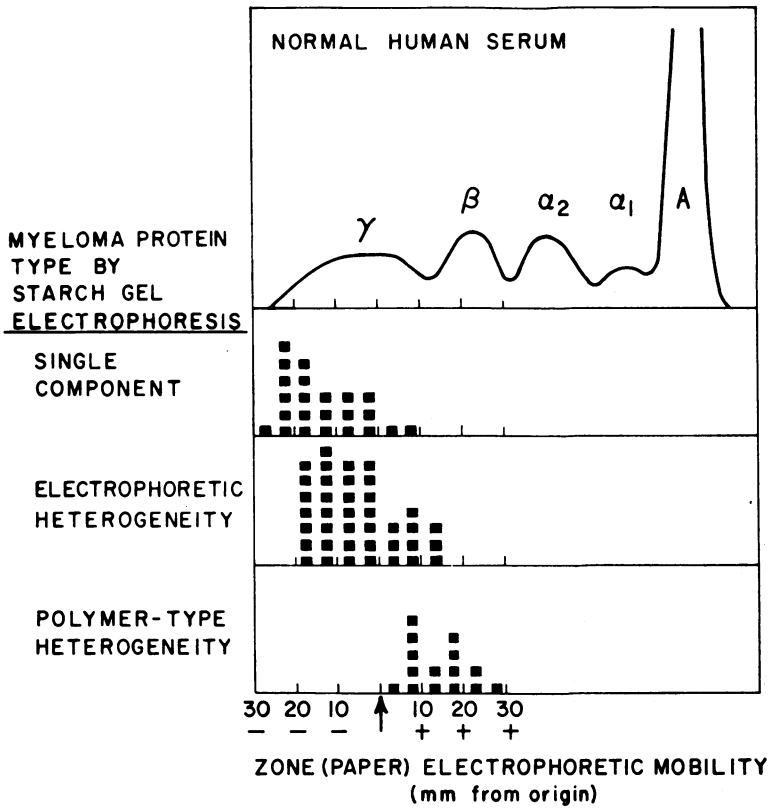

FIG. 2. ELECTROPHORETIC MOBILITY OF DIFFERENT TYPES OF MYELOMA PROTEINS. Electrophoresis was carried out on paper strips in a standard manner so that albumin migrated approximately +75 mm from the site of application (indicated by the arrow). The mobility of myeloma proteins was determined by measuring the distance from the site of application to the midpoint in the myeloma protein band. The relative mobilities of 23 myeloma proteins showing a single component on starch gel electrophoresis, 39 myeloma proteins showing electrophoretic heterogeneity, and 15 myeloma proteins showing polymer-type heterogeneity are graphically represented.

Starch gel electrophoresis was carried out by the vertical procedure of Smithies (21) modified by using discontinuous buffer system as described by Poulik (22) with ⅓ saturated NaCl in the electrode reservoir and 0.1 M Tris borate pH 9.2 buffer containing 0.02 M EDTA in the bridge chamber. The starch gel was prepared in 0.05 M pH 8.8 glycine buffer as suggested by Askonas (17). The gel was covered with Saran film during electrophoresis at 390 volts for 5 hours at room temperature. Sections of sliced gel were stained with amido black and washed with methanol: acetic acid: water (5:1:5).

Preliminary observations indicated that several factors contributed to the detection of myeloma protein heterogeneity. Dilution of sera to a myeloma protein concentration of 5 to 10 mg per ml was helpful. Glycine-NaOH buffer (17) proved especially useful in these tests, whereas preliminary trials with pH 8.6 borate buffer (21) frequently failed to reveal the multiple components in myeloma proteins with electrophoretic heterogeneity. In addition, various batches (lots) of hydrolyzed starch differed in the clarity of component resolution. Fresh sera and frozen sera, however, did not differ in their myeloma protein patterns.

Immuno-starch gel electrophoresis was based on the procedure described by Poulik (23). Part of the starch gel was stained for protein distribution. Subsequently, the starch gel strips for immunological testing were cut from the remaining gel and placed in parallel rows on 8.3 × 10 cm glass plates, and 20 to 25 ml of 1 per cent agar was poured over the plate and between the starch gel strips. After a brief cooling period, troughs were cut in the agar midway between the starch gel strips and antisera added. Photographs were obtained after precipitin lines developed, usually after 2 to 7 days.

Immunoelectrophoresis in agar was carried out by a modification of the method of Scheidegger (24). Antisera reacting specifically with 6.6S γ-globulins, β_{2A}-globulins, or γ_1-macroglobulins as well as antisera reacting with all three classes of gamma globulins were used to identify and classify serum myeloma proteins (25).

TABLE II

Ultracentrifugal analysis of sera or myeloma proteins characterized by starch gel electrophoresis in Figure 1

Sample		Ultracentrifugation			
		4S	6.6S	9S	18S
		g/100 ml			
Normal serum		5.94	0.93		0.23
Single component myeloma protein sera	1.	2.75	8.25		
	2.	3.55	8.25		
	3.*		100		
	4.*		100		
	5.	5.86	1.74		
Myeloma proteins, electrophoretic heterogeneity	6.	4.17	5.62	0.32	0.15
	7.	3.82	5.90		0.27
	8.	2.76	9.80		0.53
	9.	3.85	6.01		0.13
	10.*		100		
	11.*		100		
Myeloma proteins, polymer-type heterogeneity	12.	4.39	2.06	1.10	0.45	0.20	0.06
	13.	3.75	2.82	0.94	0.45	0.32	0.12
	14.	3.24	5.96	1.13	0.44	0.24	0.19
	15.	4.99	2.63	1.72	0.35	0.20	
	16.	4.43	5.17	0.99		0.40	0.10
	17.	5.81	1.29	0.37			0.33

* Purified myeloma proteins prepared by DEAE-cellulose chromatography and tested by immuno- and starch gel electrophoresis.

Ultracentrifugal analyses were performed in a Spinco Model E ultracentrifuge at a speed setting of 59,780 rpm with an An-D rotor and a bar or phase plate angle of 60 degrees. Sera or purified proteins were diluted with 0.14 M NaCl to a myeloma protein concentration of 0.5 to 1.0 g per cent. Photographs were obtained at 16-minute intervals after full rotor speed was reached. The relative amount of each component was calculated by planimetric measurements of enlarged tracings. Experimentally observed sedimentation coefficients were corrected to a water basis at 20° ($s_{20,w}$).

Cation-exchange cellulose chromatography was carried out on carboxymethyl(CM)-cellulose (26). Approximately 12 mg of protein was applied to each gram of adsorbent. The initial buffer composition and the condition of elution are given with each experiment. A gradient increase in ionic strength was created by using three chambers of a Varigrad (27) with equal volumes of the initial buffer in the first two chambers and the limit buffer in the third chamber. Anion-exchange cellulose chromatography was carried out on DEAE-cellulose columns as described previously (28).

RESULTS

Serum studies. Myeloma proteins were heterogeneous in 70 per cent of 77 cases of multiple myeloma (Table I). Heterogeneity was readily detected by starch gel electrophoresis and was confirmed by ultracentrifugal and chromatographic analyses. Three patterns of myeloma protein distribution were seen by starch gel electrophoresis and are illustrated in Figure 1. A single anomalous band was seen in 30 per cent of the sera. In approximately half of the sera, multiple discrete bands at closely spaced periodic intervals (electrophoretic heterogeneity, Figure 1) were seen. Finally, several widely space components (polymeric heterogeneity, Figure 1) were seen in 20 per cent of the sera.

Starch gel electrophoretic heterogeneity reflected intrinsic properties of the myeloma proteins. Purified myeloma proteins had the same starch gel electrophoretic properties as were seen in whole serum, and fresh and frozen and thawed sera showed the same patterns. The individuality of the myeloma proteins was seen in their patterns of heterogeneity. No two of the 54 heterogeneous myeloma proteins showed identical starch gel electrophoretic patterns (see Figure 1).

The electrophoretic distribution of the three forms of myeloma protein is graphically illustrated in Figure 2. Electrophoretic heterogeneity was seen in myeloma proteins migrating anodally

or cathodally from the site of sample application or origin on starch gel electrophoresis. All the examples of polymer-type heterogeneity were seen in myeloma proteins migrating towards the anode. Single component myeloma proteins were found to migrate anodally or cathodally and were represented in both γ- and β_{2A}-types of myeloma globulin.

Ultracentrifugal analysis revealed differences between the sera showing electrophoretic heterogeneity and sera showing polymer-type heterogeneity. Table II contains ultracentrifugal data on the myeloma proteins characterized by starch gel electrophoresis in Figure 1. Marked elevation of the 6.6S component is seen in the samples having electrophoretically heterogeneous myeloma proteins (Figure 3). In samples showing polymer-type heterogeneity, multiple components that sedimented at 6.6S, 9S, 11S, and 13S were seen in the ultracentrifuge (Figure 3). This characteristic difference is examined in greater detail below. Most single component myeloma proteins were associated with 6.6S myeloma proteins, although one (G.F.) was found to sediment at 9S in the ultracentrifuge.

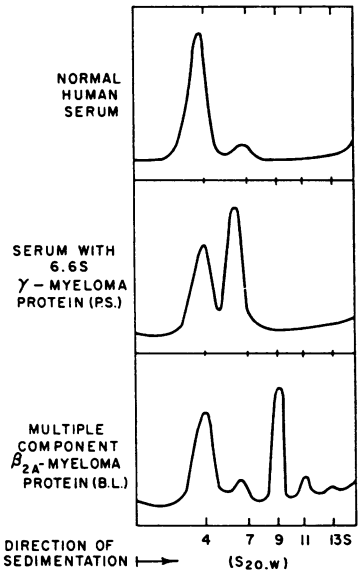

FIG. 3. ULTRACENTRIFUGAL ANALYSIS OF TWO TYPES OF HUMAN MYELOMA SERA. Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge in an An-D rotor with a 1° angle face in one cell. Sera were diluted in 0.14 M NaCl to a protein concentration of 1.0 to 1.4 g per cent. Drawings were made of photographs obtained 42 minutes after reaching full speed (59,780 rpm). Serum P.S. contained an electrophoretically heterogeneous γ-myeloma protein and serum B.L. a polymer-type β_{2A}-myeloma protein.

Immunochemical types of myeloma proteins. The type of myeloma protein heterogeneity was found to relate to the immunochemical class of myeloma protein (Table I). Each myeloma protein was classified as γ-type or β_{2A}-type on an immunochemical basis, the γ-myeloma proteins being closely related to normal 6.6S γ-globulins and the β_{2A}-myeloma proteins being related to the β_{2A}-globulins of normal serum. Immunoelectrophoretic analyses of representative myeloma sera are shown in Figure 4. Gamma-myeloma proteins are revealed by their reaction with specific anti-γ-globulin antiserum; β_{2A}-myeloma proteins by their reaction to specific anti-β_{2A}-antiserum.

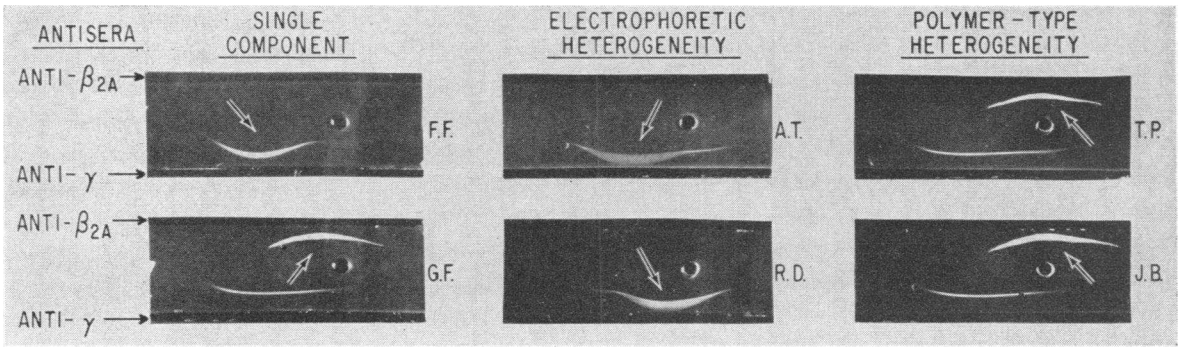

FIG. 4. IMMUNOELECTROPHORETIC CHARACTERIZATION OF SERUM MYELOMA PROTEINS SHOWING A VARIETY OF PATTERNS ON STARCH GEL ELECTROPHORESIS. After electrophoresis in agar, each serum was allowed to react with specific anti-β_{2A}-globulin antiserum (above) and specific anti-6.6S γ-globulin antiserum (below). Photographs were obtained 24 hours after electrophoresis. The arrows indicate the anomalous protein. F.F. is seen as serum no. 3 in Figure 1, G.F. is seen in Figure 8, and analyses on the remaining sera are in Figure 1: A.T. = no. 8, R.D. = no. 11, T.P. = no. 13, and J.B. = no. 15.

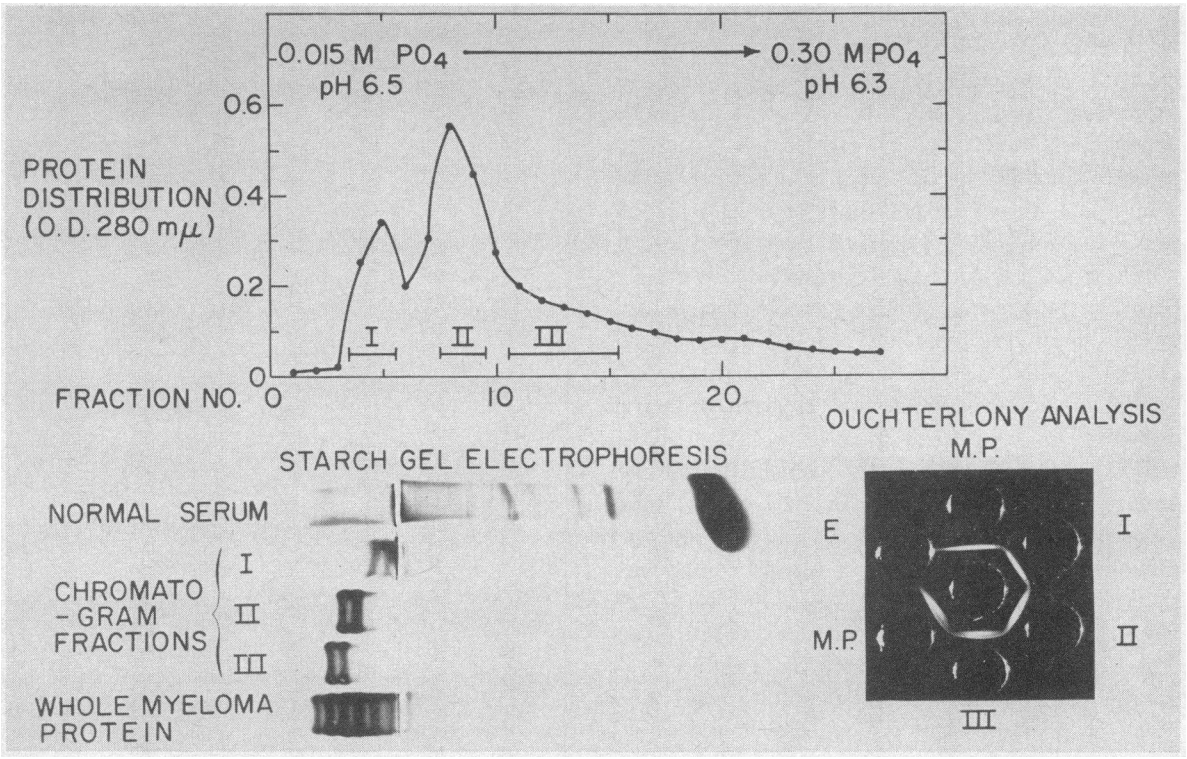

FIG. 5. CHROMATOGRAPHIC FRACTIONATION OF AN ELECTROPHORETICALLY HETEROGENEOUS γ-MYELOMA PROTEIN. The myeloma proteins were separated from the other components in A.H. serum by chromatography on a DEAE-cellulose column (not illustrated). The myeloma protein components were then subfractionated on a column of carboxymethyl cellulose with a gradient elution as illustrated. The effluent was divided into three fractions (I, II, III), concentrated by ultrafiltration, and characterized by starch gel electrophoresis as seen here and by ultracentrifugation (Table III). Immunochemical comparison of the fractions with unfractionated myeloma protein was done by gel diffusion analysis in Ouchterlony plates. The result when antinormal 6.6S γ-globulin antiserum was used in the center well is shown. M.P. = whole myeloma protein; E = empty well.

Electrophoretic heterogeneity was seen only with γ-myeloma proteins. Polymer-type heterogeneity was found only with β_{2A}-myeloma proteins. Single component myeloma proteins were found among γ- and β_{2A}-myeloma proteins.

Electrophoretic heterogeneity. The multiple components seen on starch gel electrophoresis could be partially separated by chromatography on columns of cation or anion exchanging, substituted cellulose (26). Five myeloma proteins with electrophoretic heterogeneity were studied in detail and findings with a representative myeloma protein from patient A.H. are presented in Figure 5. Myeloma protein A.H. was fractionated on a CM-cellulose column, and starch gel electrophoresis of the chromatogram fractions revealed that the first components eluted from the CM-cellulose column are the fastest migrating and that subsequent fractions contain more slowly migrating components (Figure 5). CM-cellulose chromatography was useful for fractionating slowly migrating γ-myeloma proteins. The more rapidly migrating myeloma proteins could be fractionated on DEAE-cellulose columns by applying the sample at low ionic strength and alkaline pH and eluting the proteins by gradient or stepwise increase in ionic strength (not illustrated).

TABLE III

Ultracentrifugal properties of the components from electrophoretically heterogeneous myeloma protein A.H.

Sample		Sedimentation coefficient ($s_{20,w}$)
Whole myeloma protein		7.32
CM-cellulose Chromatography Fractions	1	7.02
	2	7.12
	3	7.02

Ultracentrifugal comparison of the components of a γ-myeloma protein (separated as in Figure 5) revealed that all had approximately the same sedimentation properties, each sedimenting as a single peak with sedimentation coefficients similar to that of the unfractionated myeloma protein (Table III). This observation, repeated in four other γ-myeloma proteins, indicated that the multiple components seen on starch gel electrophoresis of γ-myeloma proteins did not differ in size. Similarly, ultracentrifugation of whole serum (Figure 3) revealed only an increase in the 6.6S component. The electrophoretic studies noted above, however, indicate that the multiple discrete bands at closely spaced periodic intervals are due to differences in the net electrical charge on the components. Thus, this type of heterogeneity is referred to as electrophoretic heterogeneity.

Immunochemical comparison of the separated components of electrophoretically heterogeneous myeloma proteins was undertaken to determine if they differed in antigenic as well as electrophoretic properties. Components of the A.H. myeloma protein were examined by the Ouchterlony double diffusion technique as shown in Figure 5. No antigenic differences could be detected, although six antisera prepared against a variety of normal gamma globulin fractions and myeloma proteins were employed in separate tests. Similarly, with two other heterogeneous myeloma proteins the reactions of antigenic identity formed by the precipitin lines indicated that the electrophoretically different components of individual myeloma proteins had the same antigenic determinants. Additional studies, showing cross-reactions as well as antigenic differences between several myeloma proteins and Bence-Jones proteins, demonstrated that several of the antisera employed in these tests reacted with five or more antigenic determinants and that four or more antigenic determinants were being detected on each myeloma protein. These observations indicate that the structural differences responsible for electrophoretic differences between myeloma protein components do not involve sites on the molecule which are antigenic in the rabbits.

Polymer-type heterogeneity. Several widely spaced myeloma protein components were found on starch gel electrophoresis of 15 sera, and several examples are shown in Figure 1. The separate protein bands were identified as components of the β_{2A}-myeloma protein by immuno-starch gel electrophoresis. In Figure 6 is shown the result after starch gel electrophoresis when the serum proteins were allowed to diffuse out of the gel strip into agar in which an antisera trough had been cut. When the trough was filled with antisera reacting specifically with β_{2A}-globulins, the antibodies diffusing from the trough and the β_{2A}-globulin components diffusing from the starch gel formed a precipitin band in the agar. Precipitin

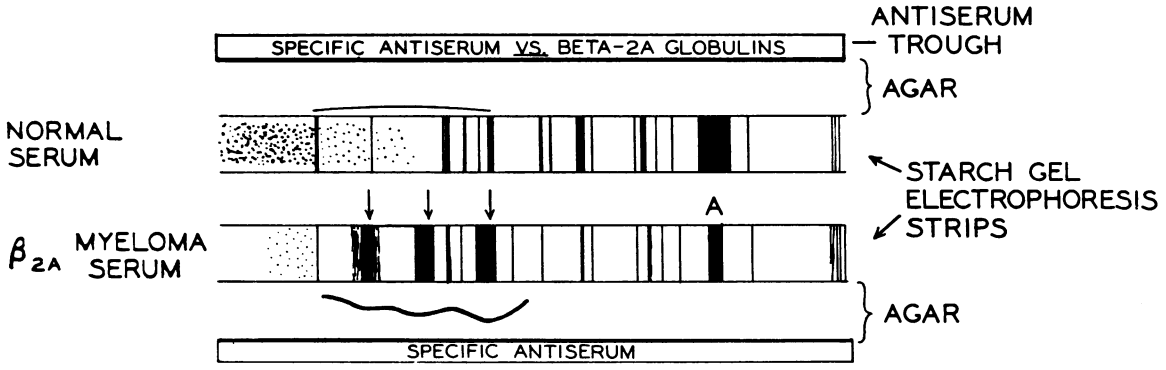

FIG. 6. STARCH GEL IMMUNOELECTROPHORESIS OF β_{2A}-MYELOMA SERUM DEMONSTRATING THAT THE ANOMALOUS BANDS IN THE SERUM ARE β_{2A}-GLOBULINS. Starch gel electrophoresis was carried out as in Figure 1. Half of the sliced gel was stained, while a strip containing the electrophoretically separated proteins was cut from the unstained half of the gel, placed on a glass plate, and surrounded with agar. When the agar hardened, a trough was cut and specific antiserum against human β_{2A}-globulins was added. After 48 to 168 hours the plates were photographed. The drawing was made by combining the findings with the stained half of the gel with the results of the immuno-starch gel test.

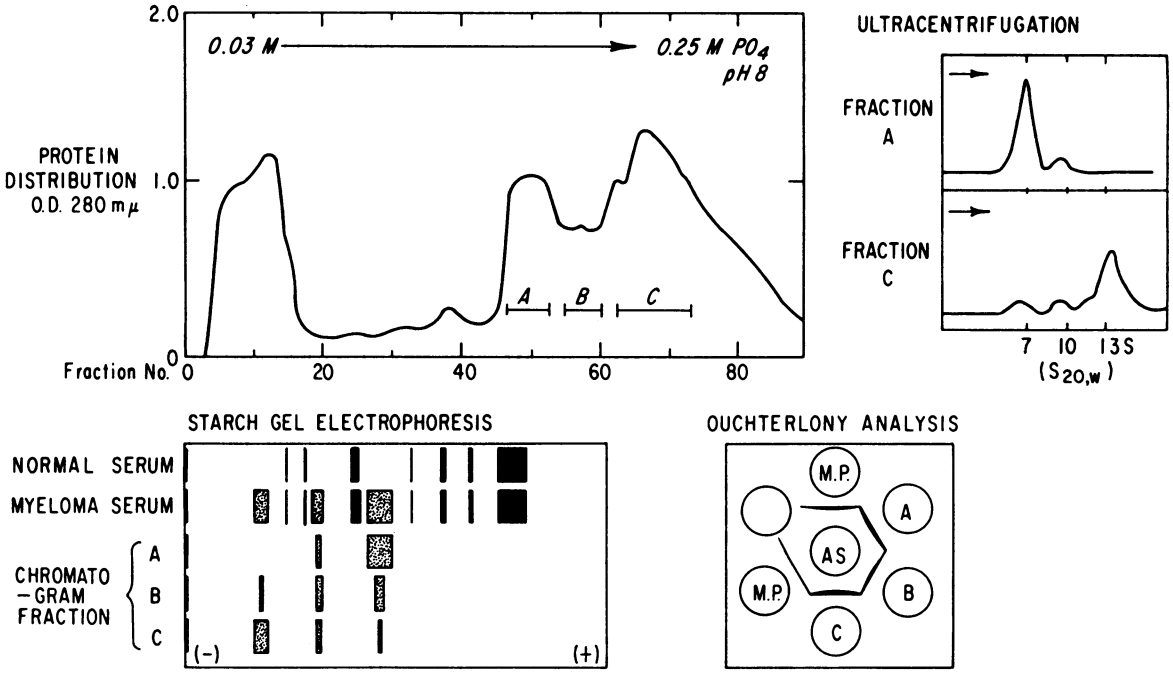

FIG. 7. CHROMATOGRAPHIC FRACTIONATION OF THE MULTIPLE COMPONENTS OF A β_{2A}-MYELOMA PROTEIN. Serum from mice bearing the SPC-1 plasma cell tumor was fractionated by zone electrophoresis on polyvinyl copolymer blocks, and 390 mg of the β-globulin fraction containing the myeloma proteins was further fractionated by chromatography on 7 g of DEAE-cellulose. Proteins were eluted by gradient elution with 0.03 M and 0.25 M phosphate pH 8 buffers as initial and final buffers. The gradient was established in a Varigrad (27) with initial buffer in chambers 1, 2, 3, 5, and 7; final buffer in 6, 8, and 9; and 4:1 mixture in chamber 4. The major chromatogram fractions, including A, B, and C, were concentrated by ultrafiltration and tested by starch gel and immunoelectrophoresis, ultracentrifugation, and Ouchterlony analyses. Ultracentrifugation was done at 5 mg per ml protein concentration and the drawings represent photographs taken 26 minutes after reaching full speed (59,780 rpm). In the Ouchterlony plate, unfractionated myeloma protein (M.P.) is compared with fractions A, B, and C by using rabbit anti-5647 myeloma protein antiserum (AS) in center well. The well in the upper left is empty.

lines developed opposite the myeloma protein bands in the starch gel. Subsequently the separate precipitin bands fused to form a single scalloped precipitin band as shown in Figure 6. This reaction served to identify the several widely spaced bands in the starch gel as components of the β_{2A}-myeloma protein.

Serum ultracentrifugal analyses indicated that the several widely spaced components seen on starch gel electrophoresis might be due to components of different sizes. All 15 sera showing polymer-type heterogeneity on starch gel electrophoresis contained components with sedimentation coefficients ($s_{20,w}$) of 9, 11, and 13S, as well as 6.6S components (Figure 3). Since starch gel exerts a filtration effect on the globulin molecules migrating through the gel matrix, it was reasoned that the components larger than 6.6S would migrate less far than the 6.6S component. Direct evidence that size differences were responsible for the multiple components seen on starch gel electrophoresis of β_{2A}-myeloma proteins was obtained by separation of the components.

Fractionation of β_{2A}-myeloma proteins was done on columns of DEAE-cellulose, and the fractions were compared by ultracentrifugation, starch gel electrophoresis, immunoelectrophoresis, and Ouchterlony double diffusion tests. In the experiment shown in Figure 7, beta globulins containing the β_{2A}-myeloma proteins from mice bearing the SPC-1 plasma cell tumor were isolated by zone electrophoresis on Pevikon polyvinyl copolymer particle blocks and then fractionated chromatographically on DEAE-cellulose columns. Two human sera containing β_{2A}-myeloma proteins were similarly fractionated but the resultant fractions contained a greater percentage of normal serum components than did sera from mice bear-

ing the SPC-1 or the X5647 plasma cell tumors. The proteins initially eluted in Figure 7 included transferrin and other normal components. Myeloma protein components began to be eluted at tube 45. The first myeloma protein component collected in fraction A migrated relatively rapidly on starch gel electrophoresis and contained largely 6.6S globulins (Figure 7). Later eluted fractions (B and C in Figure 7) contained greater amounts of 9, 11, and 13S ultracentrifugal components and more of the slower migrating components on starch gel electrophoresis. The distinct peaks formed in the ultracentrifuge by the 6.6S, 9, 11, and 13S components indicated that these molecules differed in some regular manner and that little if any heterogeneity existed within each component. The findings were also consistent with the possibility that the 9, 11, and 13S components were polymers of 6.6S molecules, as is the case in macroglobulinemia (29).

Mercaptoethanol treatment of purified myeloma proteins containing 9S components (Figure 8, G.F.) or 9, 11, and 13S components (Figure 8, C.H.) reduced all or almost all of the larger myeloma protein components to 7S. Six myeloma sera containing appreciable quantities of 9, 11, and 13S components were similarly treated with 0.1 M mercaptoethanol, and in each case 6.6S components increased and a proportional decrease occurred among the larger components. These observations indicate that 9, 11, and 13S components may be made up of smaller 6.6S protein units, which have been joined into polymers by disulfide bonds. Calculations revealed that the sedimentation coefficient of a complex of two 6.6S molecules with molecular weight of 160,000 could be about 9S. If the 9S component is formed from two 6.6S units, the 11S and 13S components may well be complexes of three and four such units.

The antigenic properties of the several components of the β_{2A}-myeloma proteins were found to be similar. On starch gel immunoelectrophoresis, as seen in Figure 6, the continuous connection between the precipitin arcs formed by each myeloma protein band indicates that the different β_{2A}-globulin components possess common antigenic determinants. Also, Ouchterlony double-diffusion comparison of components separated by chromatography, as in Figure 7, showed that the fractions of the β_{2A}-myeloma protein shared common antigenic determinants. The present studies indicate antigenic similarity between the components of different size, but a variety of antisera, including some prepared against the larger components, would have to be used in similar tests before it can be concluded that these components are antigenically identical.

DISCUSSION

Characterization of 77 myeloma proteins by starch gel electrophoresis and ultracentrifugation revealed 70 per cent of the individual myeloma proteins to be heterogeneous.

Considerable evidence indicates that most myeloma proteins are genuinely heterogeneous and that these findings are not artifactual. The demonstration of heterogeneity in fresh serum samples indicates that heterogeneity is not due to aging, nor to degradation from bacterial enzymes. The presence of heterogeneity in purified myeloma proteins and failure to detect other serum

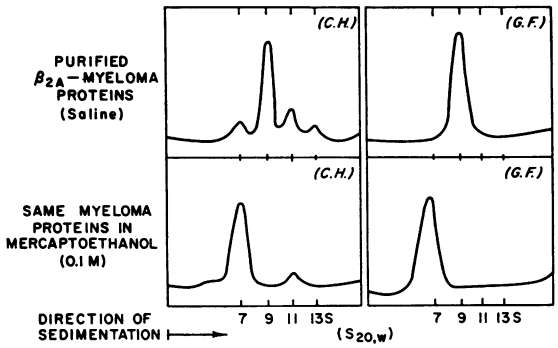

FIG. 8. REDUCTION OF 9, 11, AND 13S MYELOMA PROTEIN COMPONENTS TO 6.6S COMPONENTS BY MERCAPTOETHANOL. Representative β_{2A}-myeloma proteins were prepared from the sera of patients C.H. and G.F. by a sequence of block electrophoresis and DEAE-cellulose chromatography in a manner similar to that in Figure 7. Ultracentrifugation, carried out as in Figure 4 and illustrated in the top half of Figure 8, revealed the anomalous proteins of the whole serum to be present in the test fractions. The C.H. myeloma protein contained a mixture of 6.6, 9, 11, and 13S components. The G.F. myeloma protein had only 9S components. Dialysis of the myeloma proteins for 16 hours against 0.1 M mercaptoethanol resulted in reduction of all or almost all of the myeloma protein components to 6.6S components as shown by the ultracentrifugal analyses in the lower half of Figure 8. Photographs taken 42 minutes after reaching rotor speed (59,780 rpm).

components in the purified proteins when tested by immunochemical procedures indicate that the observed differences are not due to combination of the myeloma globulins with other serum components. In addition, the separation of components by chromatographic techniques shows that the appearance of multiple components are not artifacts of the starch gel electrophoretic procedure. Finally, the identification by Askonas (17) of the components of an electrophoretically heterogeneous myeloma protein in microsomal extracts of a mouse plasma cell tumor indicate that the components are synthesized in the malignant cells and do not represent products of partial catabolism of a single protein.

The two types of heterogeneity observed, electrophoretic and polymer-type, could be readily distinguished by their appearance on starch gel electrophoresis. Electrophoretic heterogeneity was seen as multiple, neatly banded, periodically arranged components migrating closely together. Polymer-type heterogeneity was seen as several, relatively diffuse but widely spaced bands differing in size. The two types of heterogeneity occurred in immunochemically different classes of globulins, electrophoretic heterogeneity being seen only with γ-myeloma proteins, whereas polymer-type heterogeneity was seen only with β_{2A}-myeloma proteins. A similar distinction between γ- and β_{2A}-myeloma proteins has been noted with mouse myeloma proteins (18). The ultracentrifugal studies of Laurell (30) and Imhof and Ballieux (31) also note the presence of several components that settle as sediment more rapidly than 7S in β_{2A}-myeloma sera.

The 9, 11, and 13S components seen on ultracentrifugation of the β_{2A}-myeloma sera appear to be polymers of a specific type of 6.6S component, the 9, 11, and 13S components presumably being composed of 2, 3, and 4 units, respectively. The dissociation effects of mercaptoethanol, which have also been reported recently by Deutsch (32), and the distinct separation of the 6.6S, 9, 11, and 13S components on ultracentrifugation and starch gel electrophoresis indicate that the 6.6S molecular units are associated through binding at specific sites containing SH-groups.

Beta$_{2A}$-globulin components sedimenting at 9, 11, and 13S have not been positively identified in normal human serum, although they might escape detection by the relatively insensitive ultracentrifugation techniques employed in routine serum analysis. Heremans, Heremans, and Schultze (33) reported 10 and 13S components in their concentrates of β_{2A}-globulin from normal human serum. The 9S β_{2A}-globulin is not to be confused with the 9S component routinely found in γ-globulins prepared by ethanol fractionation of human serum (34). The latter component appears to be a polymer of normal 6.6S γ-globulin and shares the antigen and chromatographic features of γ-globulins, thus differing from the 9S β_{2A}-globulins described here.

The electrophoretic heterogeneity of γ-myeloma proteins indicates that the components of individual myeloma proteins differ in net charge. It seems probable that the charge differences reflect differences in amino acid composition of the molecules. The first possibility to be considered is that substitution of an amino acid differing in charge—positive, negative, or none—at a single site in one of the polypeptide chains of a γ-myeloma protein accounts for the difference between two or, at most, three of the components. Similar net charge differences due to substitution of single amino acid of different charge have been reported in variants of hemoglobin molecules (35) and albumin molecules (36). In the case of γ-myeloma proteins, however, the frequent finding of four or more components (and as many as 10 components) indicates that amino acid interchange, if that is the sole basis for the observed heterogeneity, would have to occur at several sites in the γ-globulin molecule. Preliminary studies (37) employing enzymatic (papain) digestion of components of an electrophoretically heterogeneous mouse γ-myeloma protein indicated that the charge differences between four intact myeloma protein components were reflected in differences in the S and F fragments resulting from papain digestion, and are in accord with the postulate that the several components of a myeloma protein may differ by charge differences at several sites on the molecules.

Alternative bases for myeloma protein heterogeneity, however, have not been ruled out. Substitutions among uncharged amino acids could produce structural changes influencing starch gel electrophoretic behavior. It is also possible that

one primary amino acid sequence might occur in several conformational arrangements.

These same possibilities apply in a consideration of the polymeric heterogeneity of β_{2A}-myeloma proteins. It seems probable that the molecules that form 9S complexes differ at a specific site, either in primary structure or in conformation, from the related molecules which remain as 6.6S components and differ, too, from those that form larger (11S and 13S) combinations. Additional information on the physicochemical basis for polymer formation should be helpful in this regard.

Heterogeneity was not grossly evident in the γ-myeloma proteins described as having a single component, but other conditions of starch gel electrophoresis were not extensively explored to see if occult heterogeneity could be revealed. It was notable, however, that most of these proteins were more diffusely distributed on starch gel electrophoresis than any single component of the electrophoretically heterogeneous γ-myeloma proteins. This was not an artifact due to differences in protein concentration, for the differences were still evident when purified preparations were tested at equivalent concentrations. Whether the relatively diffuse starch gel distribution of these myeloma proteins reflects heterogeneity of molecular configuration, heterogeneity due to substitutions among noncharged amino acids, or other factors which cause a truly homogeneous myeloma protein to spread slightly on starch gel electrophoresis remains to be determined.

The cellular basis for myeloma protein heterogeneity remains to be determined for it is not clear whether the several components of a heterogeneous myeloma protein are formed in the same cell or are formed in separate cells. This question has some bearing on whether multiple myeloma is to be regarded as a malignant disease of a single clone of plasma cells metastasized to multiple sites or whether malignant transformation has occurred in multiple clones of plasma cells. This last possibility is given some support by the recent observations of Potter (38), who has shown that BALB/c mice given mineral oil or other adjuvants intraperitoneally may develop several different plasma cell neoplasms. If plasma cell tumors are composed of different cell lines, each producing a separate protein component, a stable polymorphism among the cells would be required to explain the stability in the relative amounts of individual components in patients and in mice with transplantable plasma cell tumors (39). On the other hand, if these heterogeneous proteins represented the combined products of several different tumors, the proteins would not be expected to be so closely related. The differences between myeloma proteins of two patients are more marked than the differences between myeloma protein components of any individual patient. Similar wide myeloma protein differences are observed between plasma cell tumors arising in inbred mouse strains (18), indicating that the differences are not due to host genetic factors. The finding that the several components of a myeloma protein are closely related favors the possibility that the tumor is composed of a single clone of malignant plasma cells each forming all the myeloma protein components, or that the tumor is composed of closely related clones, perhaps derived from a single plasma cell clone, existing in a stable polymorphic relationship to one another. At the present time, however, there has been no direct observation on the range of globulins synthesized in single plasma cells, normal or malignant.

Whether or not myeloma protein heterogeneity has any clinical or morphological significance is not yet known. Studies of clinical, immunologic, metabolic, and morphologic features of the disease in patients with these and other variants of myeloma are under way which, it is hoped, will shed some light on these questions.

SUMMARY

Heterogeneity was detected in 70 per cent of the myeloma proteins in sera from 77 patients. Two types of heterogeneity (electrophoretic and polymer-type) were observed and these were readily detected by starch gel electrophoresis.

Electrophoretic heterogeneity was revealed by the presence of multiple (four or more) closely spaced components arranged at periodic intervals. The myeloma protein components were partially separable by chromatography on anion- or cation-exchange cellulose columns. The separated components had similar ultracentrifugal properties and the same antigenic properties, but differed in net electric charge. Electrophoretic heterogeneity was found only in γ-type myeloma proteins.

Polymer-type heterogeneity was seen on starch gel electrophoresis as several widely spaced bands. On ultracentrifugation, 9, 11, or 13S components, or all, as well as 6.6S components, were seen in these sera. The 9, 11, and 13S components could be reduced to 6.6S by treatment with mercaptoethanol, and are believed to represent two, three, or four unit polymers, or all, composed of specific 6.6S units associated through bands involving disulfide groups. The polymers of these myeloma proteins could be partially separated by anion-exchange cellulose chromatography. They were shown to share the same antigenic determinants and were identified immunochemically as β_{2A}-globulins.

The pattern of heterogeneity was characteristic for each myeloma protein, no two patients having exactly identical patterns.

The possibilities that heterogeneity of myeloma proteins may reflect synthesis of several gamma globulin molecules in single malignant plasma cells or the coexistence of several lines of malignant cells, each producing one globulin molecule, are discussed.

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