ANTIGENIC RELATIONSHIPS OF BENCE JONES PROTEINS, MYELOMA GLOBULINS, AND NORMAL HUMAN γ-GLOBULIN*

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Various workers (1–3) have concluded that the three classes of normal immunoglobulins, 7S γ, β_{2A}, and β_{2M}, are antigenically related and are all formed in the plasma cell.[1] In abnormalities of this protein-forming cell a variety of proteins may be produced (5); for example, the γ type myeloma globulins which have been subdivided antigenically by Korngold (6, 7) into types I and II, the β_{2A} (or γ_{1A} or immunological type III) myeloma proteins, and the pathological 19S macroglobulins of the β_{2M} type. In addition, such patients may excrete Bence Jones proteins which are antigenically related to the immunoglobulins (8) and which are likewise formed in the plasma cell (9) and traverse the serum. The Bence Jones proteins have been classified antigenically by Korngold into two types, A and B (8).[2] In addition, antigenically distinct fragments of the γ-globulin–related proteins may be obtained by enzymatic cleavage with papain (11, 12). Such fragments have an s_{20} of 3.5S like that of many Bence Jones proteins and thus permit structural study of the several classes of γ-globulin–related proteins. Previous studies have revealed some of the antigenic relationships among these proteins and their 3.5S fragments, both for the human (13–15) and for the mouse (16, 17).

In the present work the interrelationships among the immunoglobulins of man have been studied by immunodiffusion and immunoelectrophoresis with respect to their relationship to the Bence Jones proteins. The results suggest that the latter may be considered as one of the possible subunits of all the immunoglobulins. The slow moving papain fragment designated S by Edelman *et al.* (13),[3] when derived from immunological type I myeloma γ-globulins, is

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[1] The evidence that the plasma cell produces macroglobulins is less secure. A peculiar lymphocyte is the main cell type in many cases of macroglobulinemia (4).

[2] Burtin (10) who originally described three antigenic types of Bence Jones proteins has revised his classification to two types in which his type I and II are equivalent to Korngold's B and A, respectively (personal communication).

[3] The 3.5S fragments of human 7S γ-globulin have been given different designations in various laboratories. It is difficult to equate them because somewhat different conditions of digestion, fractionation, and characterization have been employed, as well as different starting preparations of γ-globulin. However, there are two main precipitin lines in agar gel immuno-

antigenically very related to type B Bence Jones proteins, and the papain fragment S of type II myeloma γ-globulins is antigenically very similar to type A Bence Jones proteins. From study of various combinations of normal γ-globulin, myeloma globulins, their papain cleavage products, and both types of Bence Jones proteins with specific antisera, the immunological relationships among these proteins can be presented schematically in the form of an antigenic map.

Materials and Methods

1. Antigens.—

(*a*) *Normal gamma globulin:* The plasma immune serum globulin used (Cohn fraction II, Lot 45875 from Merck Sharp and Dohme Research Laboratory, West Point, Pennsylvania) was a highly purified 7S γ-globulin containing a trace amount of β_{2M} in immunoelectrophoresis. The latter was removed by chromatography on CM (carboxymethyl)-cellulose prior to papain cleavage or further study.

(*b*) *Myeloma proteins:* Ten samples of gamma type myeloma globulins (Ag., Si., Wi., Th., La., Bi., Ko., Po., Pe., and Ha.)[4] and five "micromolecular" (*i.e.* low molecular weight) myeloma serum proteins (Jh., Bb., Lo., Go., and Pr.) were prepared by salting-out fractionation with 1.8 M ammonium sulfate and were successively purified by starch zone electrophoresis or by DEAE (diethylaminoethyl)-cellulose chromatography (18). Four samples of β_{2A} myeloma proteins (Ln., No., Sil., and Mo.) were purified by twice repeated precipitation with 1.8 M ammonium sulfate, and then by discarding the precipitate formed in 0.1 M zinc sulfate and also the euglobulin precipitate formed on dialysis against distilled water. In most of the proteins one component was confirmed by immunoelectrophoresis and by Tiselius or paper electrophoresis; however, trace amounts of impurities were observed by immunoelectrophoresis in four samples (Ag., Si., Mo., and Jh.). Physical, chemical, and some immunological properties of seven of the eighteen proteins have previously been reported (19). Some physicochemical and immunological properties of these proteins are listed in Table I.

(*c*) *Macroglobulins:* Two samples of pathologic macroglobulins[5] (Na. and Re.) were prepared by repeated precipitation of the euglobulin fraction by dilution with ten times the volume of distilled water and dissolving the precipitate with saline.

(*d*) *Bence Jones proteins:* Thirty-nine samples of Bence Jones proteins were prepared by ammonium sulfate precipitation, in some cases followed by CM and DEAE-cellulose

electrophoresis—the slow moving or S component and the fast moving or F. From this property, the antigenic relationships to Bence Jones proteins, and the presence of genetic markers, the S component of Edelman *et al.* (13) may be identified with the A component of Hsiao and Putnam (12) and with fractions A and C of Franklin (14). Similarly, F is analogous to fragment B of Hsiao and Putnam (12) and also to the B fraction of Franklin (14). The papain fractions referred to herein are defined S and F on the basis of immunoelectrophoretic mobility. The S and F fragments can be further separated by chromatography and give multiple bands in starch gel electrophoresis (see the text). In some respects the F component is analogous to fraction III of rabbit γ-globulin antibody, and S is like fractions I and II, which have the antibody valence sites (11).

[4] Samples Ko., Pe., Lo., Go., Pr., and Sil. were kindly given by Dr. E. F. Osserman and Dr. K. Takatsuki, Department of Medicine, Columbia University, New York. Some of the Bence Jones proteins were also received from Dr. Osserman.

[5] Kindly given by Dr. R. T. Smith of the Department of Pediatrics, University of Florida College of Medicine, Gainesville.

chromatography or starch zone electrophoresis. Most of the Bence Jones proteins showed a single narrow peak on analysis by Tiselius or paper electrophoresis and by ultracentrifugation, but some of the proteins were less homogeneous and produced a broad peak. As we have described elsewhere (20), all the Bence Jones proteins were heterogeneous by starch gel electrophoresis. Immunoelectrophoresis with anti-human γ-globulin usually yielded a single precipitin line characteristic of Bence Jones protein, but sometimes horse antiserum to human

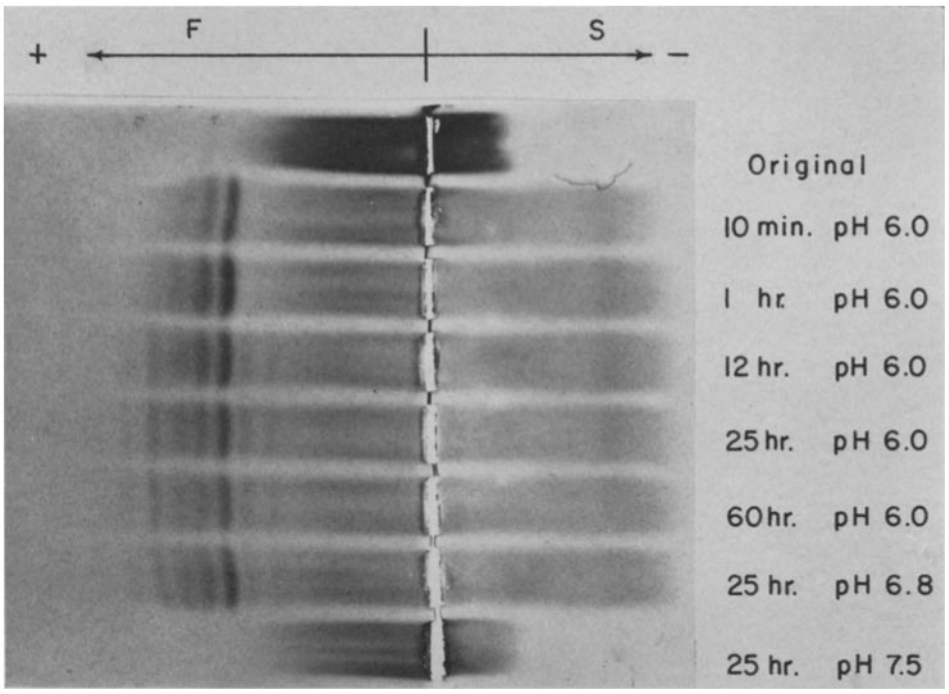

FIG. 1. Vertical starch gel electrophoresis at pH 8.65 for 5 hours of papain digests of normal human γ-globulin after incubation for different times in 0.01 M phosphate buffer of various pH's. The arrows indicate the direction of migration and the vertical line the starting slot. *S* and *F* refer to the "slow" and "fast" components described in the text.

serum[5] produced additional precipitin lines indicating the presence of albumin and γ-globulin as impurities. Some of the Bence Jones proteins have been previously reported on (21).

(*e*) *Papain-digested fragments of normal γ-globulin, myeloma proteins, and macroglobulins:* The procedure for preparation of 3.5S fragments was based on the method of Porter (11) with modifications for human γ-globulin as previously described by Hsiao and Putnam (12). One normal γ-globulin, six myeloma globulins (Ag., Wi., Th., Po., Ln., and Si.), and one macroglobulin (Na.) were digested by mercuripapain activated in 0.001 M cysteine—0.002 M EDTA at 1 per cent protein concentration and at a protein to enzyme ratio of 100:1. In some instances, the digestion time and the pH were varied in order to ascertain the optimum conditions. As is illustrated in Fig. 1, considerable undigested material was still noticed after 16 hours incubation at pH 7.5 or for shorter times at the optimal pH (5.5 to 6.5). After incuba-

tion at pH 6.0 for 16 to 25 hours, the conversion to 3.5S fragments appeared to be complete for most of the proteins as indicated by ultracentrifuge analysis, and usually no unsplit material was observed by starch gel electrophoresis or immunoelectrophoresis (Figs. 2 and 3). However, the macroglobulin (Na.) and the cryoglobulin (Th.) were difficult to dissolve and still contained undigested, or intermediate, or aggregated material. Thus, for immunization with 3.5S fragments of the macroglobulin, the protein (Na.) was digested at pH 6 for 36 hours, at the end of which time no unsplit protein was detectable by immunoelectrophoresis or ultracentrifugation.

2. Antisera.—Antisera against normal γ-globulin, one type I myeloma protein (Wi.), one β_{2A} type myeloma protein (Ln.), two macroglobulins (Na., Re.), the papain digests of normal γ-globulin and a macroglobulin (Na.), and the isolated S fragment of a type II myeloma Protein (Po.)[6] were prepared by intramuscular injection in each case into two rabbits with use of Freund adjuvant (Difco Laboratories, Inc., Detroit). A total of 40 to 150 mg of antigen was used before the first bleeding; sometimes the injections were continued to get a higher titer of the antisera. For the preparation of antisera against Bence Jones proteins, ten specimens were used as immunizing antigens (Lo., Bo., Ki., Ha., Sh., Ma., Oh., Ta., and Lo. + Sh.). Because of the poor antigenicity of Bence Jones proteins, 150 to 400 mg of the protein was injected over a 2 month period with complete Freund adjuvant; 30 to 50 mg of protein were injected each time at 2-week intervals.

3. Analytical Techniques.—The Ouchterlony immunodiffusion test was performed in 0.9 per cent agar (Ionagar, Oxoid Division, Consolidated Laboratories, Chicago Heights, Illinois) containing 0.01 per cent merthiolate in 0.9 per cent NaCl in 9 cm diameter petri dishes. Immunoelectrophoresis on a microscopic slide glass (23) or on a lantern slide coverglass was done in 1 per cent agar in 0.03 M veronal buffer, pH 8.6. Immunodiffusion reaction photographs of the precipitin lines were taken by direct print of the wet agar plate on photographic paper (kodabromide F5). The specimens were then washed with saline, dried on the glass, and stained with amido black 10B. Running water was found adequate to wash out the excess dye instead of the usual mixture of methanol, acetic acid, and water (45:10:45). In some cases photographs were taken from these stained specimens. Quantitative precipitin reactions were performed with use of 0.25 ml of antiserum and various amounts of antigen with measurement of the precipitate by ultraviolet light absorption. Starch gel electrophoresis was carried out with the vertical type of apparatus according to Smithies (24) with use of the pH 8.65 tris-citrate-borate discontinuous buffer system of Poulik (25); the current was applied for 5 hours at 200 volts across the gel. Ultracentrifuge analysis and electrophoresis both by Tiscelius method and the paper method were performed with Spinco apparatus.

RESULTS

1. Electrophoretic Heterogeneity of the S and F Components of Normal and Myeloma 7S γ-Globulins.—As demonstrated herein (Fig. 3) and previously (1, 2, 13–15), papain digests of normal and myeloma 7S γ-globulins yield two major antigenically distinct components in immunoelectrophoresis, designated S and F. The digests may also be separated chromatographically into two or three fractions according to the conditions.[3] Horizontal starch gel electrophoresis at pH 5.5 of the papain-split products of normal human γ-globulin showed that the chromatographic components A (antigenically equivalent to S) and B (antigenically equivalent to F) had different though overlapping

[6] This S fragment was isolated chromatographically after "spontaneous" cleavage of the myeloma globulin during purification. This phenomenon will be described elsewhere (22).

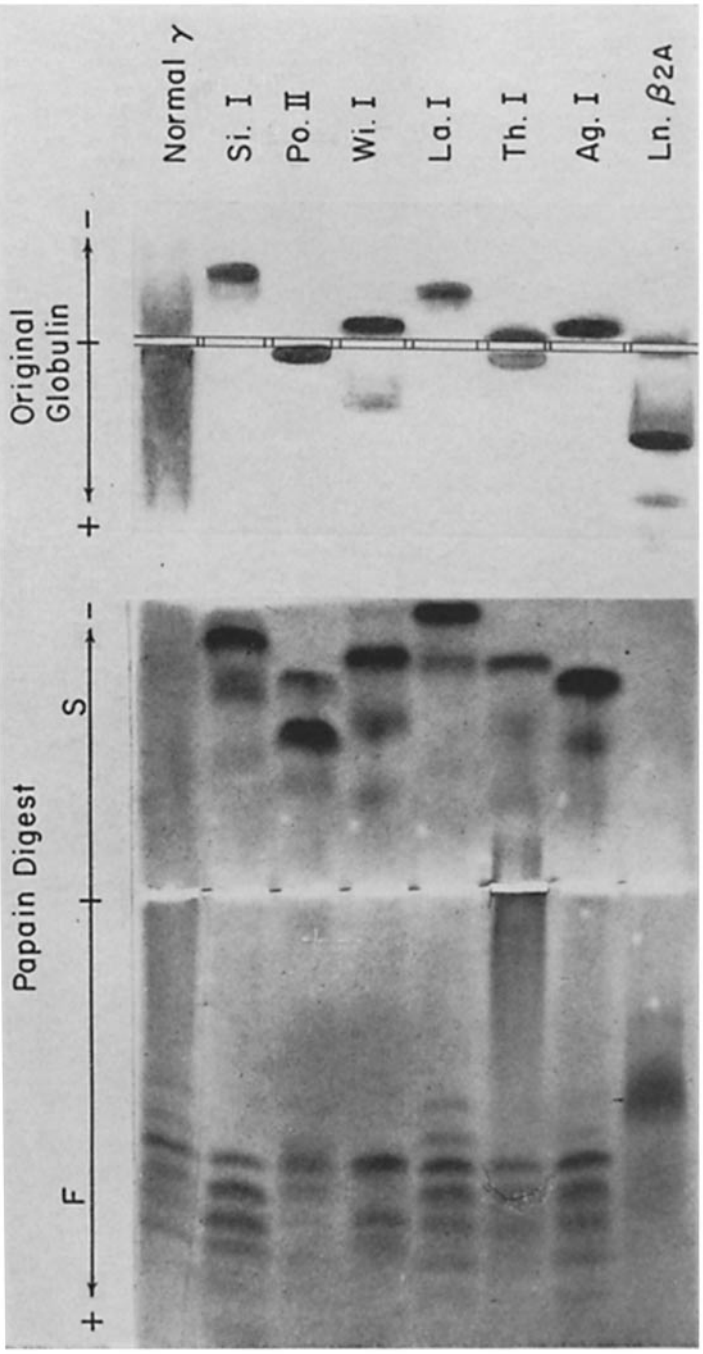

FIG. 2. Vertical starch gel electrophoresis at pH 8.65 for 6 hours of papain digests of normal human γ-globulin, five type I myeloma globulins, one type II myeloma globulin, and one β_{2A} type myeloma globulin. The digestion was performed for 25 hours in 0.01 M pH 6.0 phosphate buffer in the presence of mercuripapain, cysteine, and EDTA. On the right is a sketch of the patterns for the undigested proteins after 5 hours' electrophoresis and also the designation of the specimen and its immunological type. For the mobility in free electrophoresis, see Table I.

electrophoretic mobilities. The present results demonstrated that vertical starch gel electrophoresis in the Poulik discontinuous buffer system of pH 8.65 resolves the F component of both normal and myeloma 7S γ-globulins into a series of discrete periodic bands indicative of electrophoretic hetero-

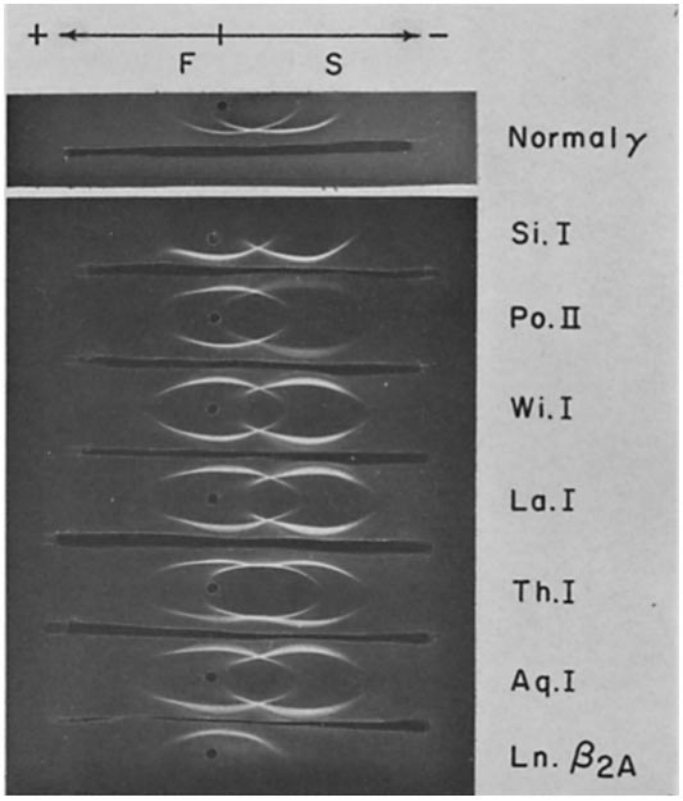

FIG. 3. Immunoelectrophoresis at pH 8.6 of papain digests of normal γ-globulin and of seven myeloma globulins shown in the same order as illustrated in Fig. 2. Rabbit antiserum against normal human γ-globulin was applied to the troughs. Th., a cryoglobulin of immunological type I, still contained undigested protein after incubation for 25 hours at pH 6 (see Fig. 2).

geneity (Figs. 1 and 2). Although the S component of normal γ-globulin is diffuse in the same system, the S components of types I and II myeloma globulins form several bands of different density and mobility. This phenomenon was not shown by the papain digest of a β_{2A} myeloma globulin, but was observed repeatedly with the papain cleavage products of a macroglobulin (Na.). An extensive study was made in the case of the macroglobulin (26); starch gel immunoelectrophoresis demonstrated that the multiple bands were

antigenically similar. Other experiments like those in Fig. 1 for normal 7S γ-globulin verified that the appearance of the multiple bands was not a function of pH, time of incubation, or the preparation of mercuripapain employed. The phenomenon may be quite general since three analogous starch gel bands are found in fraction III of the papain digest of rabbit γ-globulin (27) and multiple bands appear in the enzymatically obtained subunits of mouse myeloma globulins (16, 17).

2. The Two Immunological Types of Bence Jones Proteins.—The existence of two different types of Bence Jones protein was confirmed by use of the Ouchterlony immunodiffusion method with antiserum to normal human γ-globulin (Fig. 4, center) or to normal human γ-globulin digested by papain. The precipitin lines of the two types of Bence Jones proteins crossed each other in all cases, indicating that the two types were antigenically distinct. Furthermore, with specific antiserum against Bence Jones proteins the two different antigenic types were readily identified as shown in the following examples. An antiserum against type A Bence Jones protein that was obtained from a rabbit injected with 400 mg of Bence Jones protein (Ki.) reacted only with type A Bence Jones proteins (Fig. 4, left). The other four antisera against type A Bence Jones protein (Ta., Jh., Ha., and Sh.) that were obtained after the immunization with 150 to 300 mg of Bence Jones protein also reacted with type A Bence Jones proteins but to a varying degree. Three of them (Jh., Ha., and Sh.) cross-reacted with serum impurities but not with type B protein at all (Fig. 5). The other three antisera against type A Bence Jones protein (Oh., Mk., and Bo.) were of low titer. Four antisera—one against a type B Bence Jones protein (Lo.), another against type I myeloma globulin (Wi.), a third against a macroglobulin (Na.), and the fourth against the papain digest of the macroglobulin (ND)—all reacted only with type B Bence Jones proteins but not with type A protein, as shown in Figs. 4 (right) and 5; however, anti-Lo. also reacted with albumin present as an impurity. Another antiserum anti-(Lo. + Sh.), which was obtained by successive immunizations, first with a type B and then with a type A protein (200 mg each), reacted with both antigenic types of Bence Jones proteins (Fig. 5) (as did anti-normal γ-globulin and its papain digest). However, the antiserum obtained from the same rabbit after the first course of injection with the Lo. Bence Jones protein reacted with only type B proteins.

As shown in Table I, all Bence Jones proteins studied were easily classified into either of the two immunological types by using anti-normal γ-globulin or the specific antisera mentioned here. There were no atypical Bence Jones proteins; nor were there any discrepancies in the classification, which depended upon the choice of antisera.

Quantitative precipitin tests performed in these cases yielded results similar to the Ouchterlony immunodiffusion test (Fig. 6). Anti-normal γ-globulin

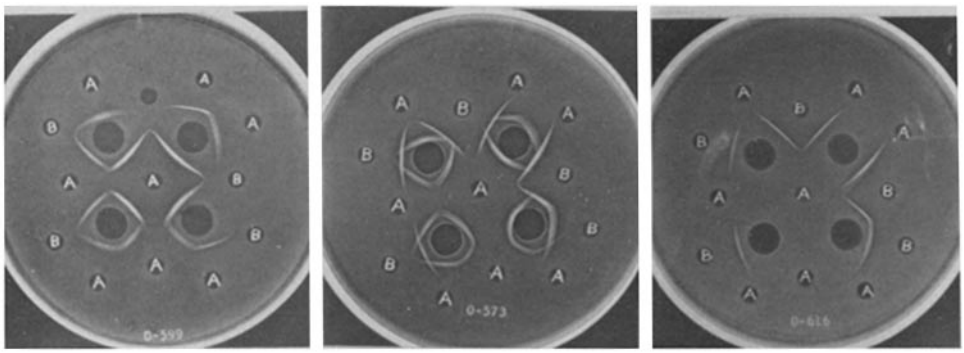

FIG. 4. Ouchterlony tests of thirteen different Bence Jones proteins with antigenic types as indicated in the photograph. The four large wells in each plate were filled with antiserum as follows: anti-type A Bence Jones protein on the left, anti-normal γ-globulin in the center, and anti-macroglobulin digest (ND) on the right.

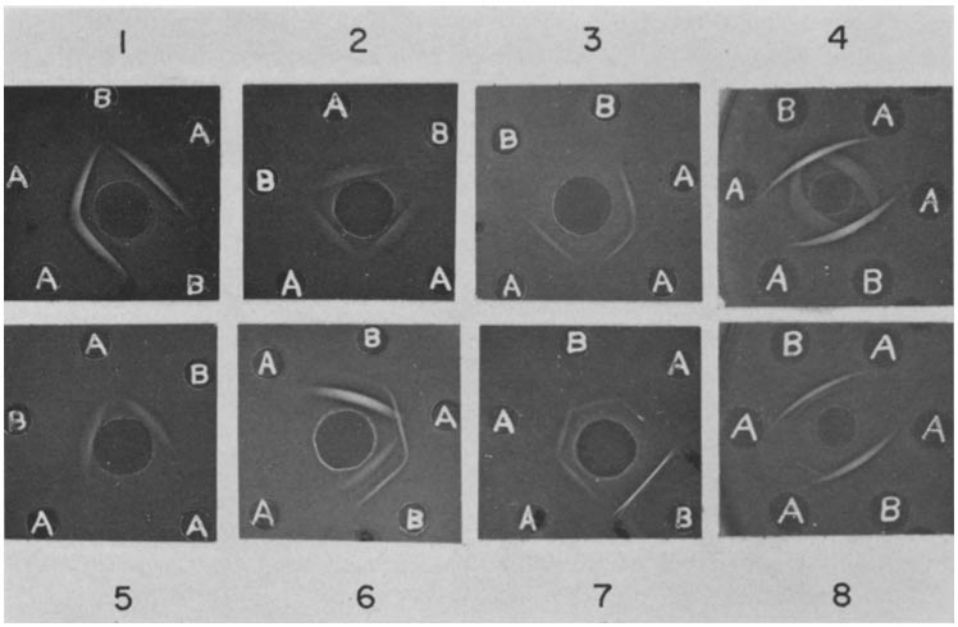

FIG. 5. Ouchterlony tests of Bence Jones proteins with specific antiserum to Bence Jones proteins. Five to six different Bence Jones proteins were placed in the surrounding wells with the antigenic type as indicated. Antiserum in the central wells in each plate was as follows: 1, anti-type A Bence Jones protein (Ta); 2, anti-type A Bence Jones protein (Jh.); 3, anti-type A Bence Jones protein (Sh.); 4, anti-digested normal γ-globulin; 5, anti-macroglobulin (Na.); 6, anti-type B Bence Jones protein (Lo.); 7, anti-types A and B Bence Jones protein (Lo + Sh.); 8, anti-type I myeloma globulin (Wi.).

TABLE I

Physical and Immunological Properties of Purified Myeloma Globulins, Macroglobulins, and Bence Jones Proteins

A. Bence Jones proteins of antigenic type A

Patient	$s_{20,w}$	Mobility	Starch gel	Serum type	Patient	$s_{20,w}$	Mobility	Starch gel	Serum type
Bb.					Kl.		2.2	3‡	γ
Be.	3.4	2.5	*	N	Lu.	3.4	2.5	9	
Bo.	3.4	2.6	6*	N	Mk.	3.4	4.7	4	β
Br.	3.5	1.2			Oh.	3.4	4.6	5	BJ-A
Go.	3.4	α_2		BJ-A	Pe.		β	*	II
Ha.	3.8	0.7	1	II	Pr.		2.2	9‡	
He.	2.5	3.4		γ	Sh.		γ	3–12	
Ht.	3.5	4.3	1		She.				
Hu.	3.4	2.6	6*	N	Su.	3.4	1.4	2	
Jh.	3.4	3.3	10*, ‡	BJ-A	Ta.	3.4	1.9	6	N
Ke.	3.2	4.2	3	N	Nd.				N
Ki.	3.3	β	4	II					

B. Bence Jones proteins of antigenic type B

Patient	$s_{20,w}$	Mobility	Starch gel	Serum type	Patient	$s_{20,w}$	Mobility	Starch gel	Serum type
Ag.	2.1	2.2	3*	I	Lo.	3.6	3.2	4	BJ-B
Bi.			8	I	Ma.	2.3	4.0		N
Bg.	3.7	3.3	2*	N	Mo.	3.1	3.4	4*	β_{2A}
Hi.				N	No.			*	β_{2A}
Ho.			3		Pi.			4	
Ko.				I	Re.	3.1	3.0	3‡	
Li.					Sil.				
Ln.	2.0		3	β_{2A}	Te.		2.3		N

C. Myeloma globulins

Patient	Antigenic type	$s_{20,w}$	Mobility	Patient	Antigenic type	$s_{20,w}$	Mobility
Ag.	I	6.6	1.1	Ln.	β_{2A}	6, 9, 11	β
Si.	I	6.4	0.8	No.	β_{2A}	6, 9, 11	β
Wi.	I	7.0	1.1	Mo.	β_{2A}	6, 9, 12	2.4
Th.	I	6.2	1.1	Sil.	β_{2A}		γ_1
La.	I	6.4	0.7	Na.	β_{2M}	18, 26, 33	1.0
Bi.	I		γ_2	Re.	β_{2M}	7, 18, 26	2.0
Ko.	I	6.7	γ_2	Jh.	A	3.8	β
Po.	II	6.3	Slow γ	Go.	A	4.1	β
Ha.	II	6.1	β	Pr.	A		γ
Pe.	II	6.6	γ_2	Bb.	A		β
				Lo.	B	3.8	β

$s_{20,w}$ is given in Svedberg units. The mobility is expressed in units of 10^{-5} cm^2 sec.$^{-1}$ volt^{-1} at 0° in pH 8.6 veronal buffer of 0.1 ionic strength except for cases where only the relative mobility was obtained by paper electrophoresis, *e.g.*, γ, β, etc. For Bence Jones proteins the number of components in starch gel electrophoresis is given for the discontinuous buffer system. The serum type, where known, is indicated either as the electrophoretic type, *e.g.*, N ("normal"), γ, or β or the immunological type, *e.g.*, I, II, β_{2A}, and β_{2M} for the globulins and BJ-A or BJ-B for "micromolecular" serum components which had the $s_{20,w}$ or antigenic characteristics of Bence Jones proteins of antigenic types A or B.

* Albumin present as an impurity.

‡ γ-Globulin present as an impurity.

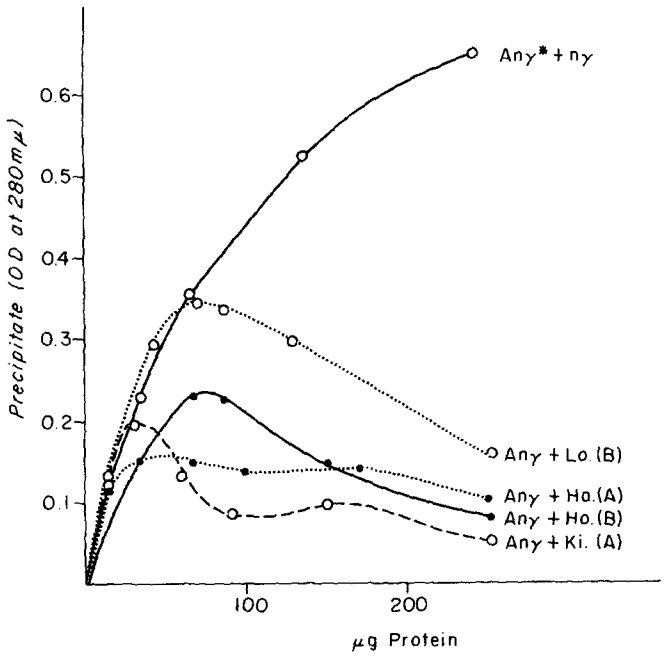

FIG. 6 *a*

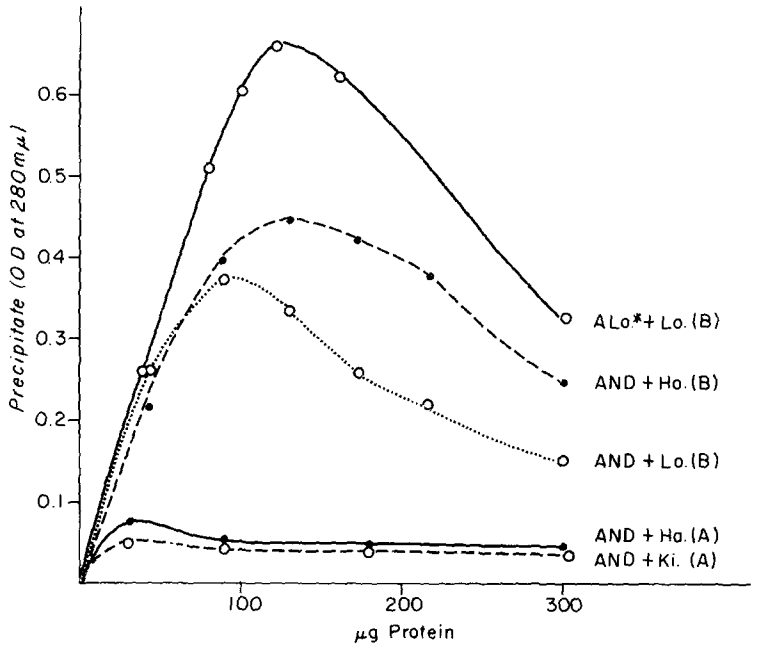

FIG. 6 *b*

FIG. 6. Quantitative precipitin curves of Bence Jones proteins with anti-normal γ-globulin (Fig. 6 *a*), with specific antiserum to type B Bence Jones protein (Fig. 6 *b*), and with specific antiserum to type A Bence Jones protein (Fig. 6 *c*). The abscissa represents the amount of Bence Jones protein added, and the ordinate gives the optical density of 280 mμ of the precipitate. In all experiments 0.25 ml of antiserum was used except those starred with an asterisk, in which case 0.125 ml of antiserum was used. The legend in the figures lists the antigen-antibody system and the antigenic type of the Bence Jones protein as established by the immunodiffusion method.

reacted with both type A and type B proteins in varying degree. The amount of precipitate produced by Bence Jones proteins was far less than that of normal γ-globulin (Fig. 6 *a*). Specific antiserum to type B protein reacted only with type B proteins (Fig. 6 *b*). Likewise, specific antiserum to type A protein gave no cross-reaction with type B protein (Fig. 6 *c*). However, three specific antisera tested produced double peaks in the precipitin curve with type A proteins. The second peak may correspond to the faint precipitin line that sometimes appears in type A proteins in the Ouchterlony test. The specificity of the sera was confirmed by absorption tests.

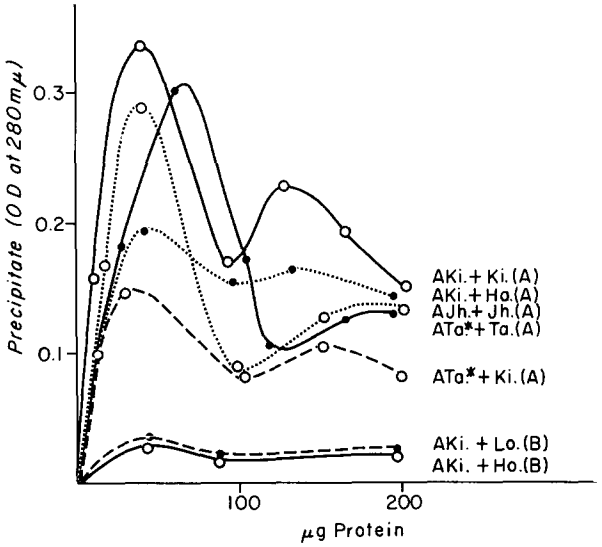

FIG. 6 *c*

3. Antigenic Relationships between Normal γ-Globulin and Bence Jones Proteins.—Papain-digested normal γ-globulin was compared with Bence Jones proteins of types A and B by immunoelectrophoresis as shown in Fig. 7. Specific antisera to type B protein and to the mixture of types A and B proteins described previously produced a precipitin line with the S fragment but with less intensity than that of anti-normal γ-globulin. Antisera to type A protein reacted with neither the S or F fragments in spite of the fact that the same antisera produced a faint precipitin line with undigested normal γ-globulin. By use of Osserman's technique (28), some antigenic determinants were found to be in common for type B protein and the S fragment. However, no relationship was found between type A proteins and either the S or F fragments. When a mixture of a Bence Jones protein of either type and papain-digested normal γ-globulin was applied as the sample in immunoelectrophoresis, the precipitin

line of the F fragment crossed or was parallel to the precipitin line of the Bence Jones protein, depending upon the mobility of the latter (Fig. 7). This indicated that no relationship exists between the F fragment and either type of Bence Jones protein. With anti-normal γ-globulin serum the precipitin line of the S fragment made a spur with that of type B protein; however, with type A proteins the S fragment made a single or double spur depending upon the Bence Jones protein used. Absorption of anti-normal γ-globulin with a type B protein

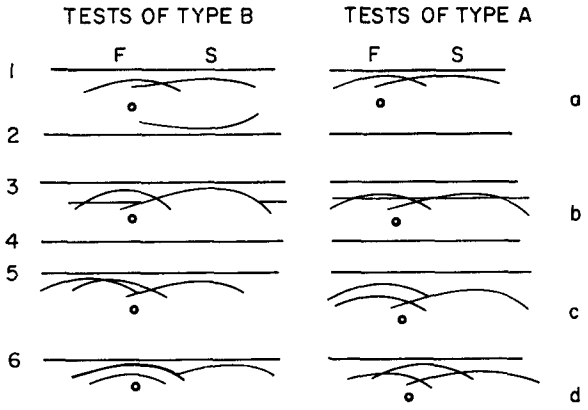

FIG. 7. Diagrams of immunoelectrophoresis of papain-digested normal γ-globulin compared to type A and type B Bence Jones proteins. In 1, 3, 5, and 6 anti-normal γ-globulin was placed in the trough marked by the numeral. In 2 anti-type B Bence Jones protein was placed in the trough to the left and anti-type A Bence Jones protein to the right. In 4 type B (Lo.) Bence Jones protein was placed in the trough to the left and Type A (Ki.) to the right, as in the Osserman technique (28). In *a* and *b* the antigen was a papain digest of normal γ-globulin. In *c* and *d* a Bence Jones protein of type B (Lo., Ag.) or of type A (Ki., Ta.), respectively, was added to the digest of normal γ-globulin. *F* and *S* indicate the position of the fast and slow precipitin lines of the papain digest.

reduced somewhat the intensity of the S precipitin line, but no change was noticed after the absorption with type A protein.

Since each Bence Jones protein has a rather limited set of antigenic determinants and also because individual differences are found among Bence Jones proteins, the successive absorption of anti-normal γ-globulin with several Bence Jones proteins was carried out as shown in Fig. 8. The precipitin line of the fast moving component (F) was not affected at all after the absorption; however, the precipitin line of the slow moving (S) became more faint with the progress of absorption of a mixture of the Bence Jones proteins of types A and B. As the extensive absorption of anti-normal γ-globulin with one type of Bence Jones protein did not change the precipitin line of the slow moving fraction very much, it may be concluded that type A and type B Bence Jones

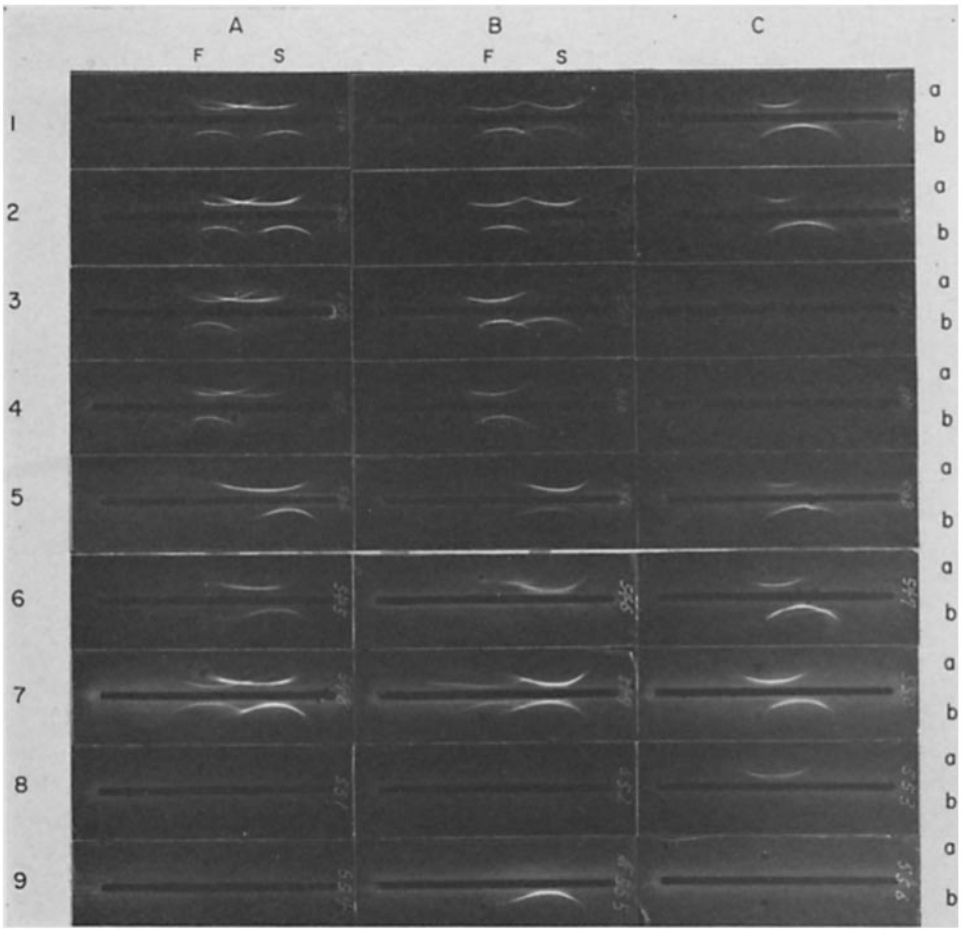

FIG. 8. Immunoelectrophoresis of papain-digested immunoglobulins with various antisera as follows:

Antigen

Aa, normal γ-globulin digested by papain
Ab, type I myeloma globulin (Wi.) digested by papain
Ba, type I myeloma globulin (Si.) digested by papain
Bb, type II myeloma globulin (Po.) digested by papain
Ca, β_{2A} myeloma globulin (Ln.) digested by papain
Cb, β_{2M} macroglobulin (Na.) digested by papain

The same arrangement of antigens is used in all photos below *A*, *B*, and *C*.

Antiserum

1, anti-normal γ-globulin
2, anti-normal γ-globulin absorbed with a mixture of type A Bence Jones proteins
3, anti-normal γ-globulin absorbed with a mixture of type B Bence Jones proteins
4, anti-normal γ-globulin absorbed with a mixture of type A and B Bence Jones proteins
5, anti-normal γ-globulin absorbed with the *F* fragment of normal γ-globulin
6, anti-digested macroglobulin (AND)
7, anti-digested normal γ-globulin (ADnγ)
8, anti-β_{2A} myeloma globulin (ALn)
9, anti-*S* of type II myeloma globulin (ASPo)

For example, in the immunoelectrophoresis slide *B3*, the antiserum is anti-normal γ-globulin absorbed with a mixture of type B Bence Jones proteins. The upper antigen (*B3a*) is a type I myeloma globulin (Si.) digested with papain, in which the *S* fragment fails to precipitate. The lower antigen (*B3b*) is a type II myeloma globulin (Po.) digested with papain, and both *S* and *F* lines appear. For absorption a Bence Jones from a different patient was used in each instance to obtain a more general result.

proteins are both related to the slow moving fraction. Nevertheless, type B Bence Jones proteins have more antigenic determinants in common with the slow moving fraction than do Bence Jones proteins of type A.

4. Antigenic Relationships between 7S Myeloma γ-Globulins and the Two Types of Bence Jones Proteins.—Ten samples of purified gamma type myeloma globulins were classified into type I and type II by means of the Ouchterlony test (Fig. 9) as listed in Table I. Normal γ-globulin produced a spur with the type I proteins and a big spur with all the type II proteins. However, as described by Korngold (6), the lines for types I and II made a double spur (*i.e.* crossed).

In immunoelectrophoresis with anti-normal γ-globulin antiserum, papain-digested myeloma globulins of types I and II both produced two precipitin

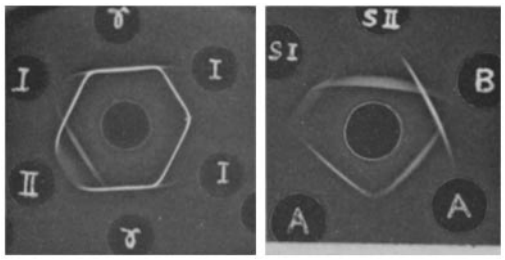

FIG. 9. Left: Ouchterlony immunodiffusion test of normal γ-globulin, and 7S-γ myeloma globulins of type I and type II with anti-normal γ-globulin. Right: *S* fragment of type I and type II myeloma globulin and three Bence Jones proteins of type A and type B with anti-normal γ-globulin.

lines like the S and F bands of normal γ-globulin, though the S precipitin line of type II is fainter than that of type I (Fig. 8 *A1b vs. B1b*). Similarly, the papain digests of both normal γ-globulin and the myeloma globulins produced two sets of distinct bands—one corresponding to the fast and the other to the slow fragment in starch gel electrophoresis (Fig. 2). The bands were spaced periodically especially in the F fragment; however, the patterns were not identical because some bands were missing in the F components, and the S bands varied in position. Although no distinct difference was observed between the two antigenic types of myeloma globulins by either starch gel or immunoelectrophoresis in the standard procedure, by use of the several identification procedures of immunoelectrophoresis previously described, the difference between type I and type II myeloma proteins was observed as follows: with specific antisera such as anti-type B (Lo.) or anti-macroglobulin digest (ND), only the slow moving fraction of type I myeloma proteins produced a precipitin line (Fig. 8 *A6b*). However, the slow moving fraction of type II myeloma proteins did not react at all (Fig. 8 *B6b*). The absorption experiment was more effective in this case. Anti-normal γ-globulin absorbed with a mixture of type

A Bence Jones proteins removed the precipitin line of the slow component of type II myeloma globulin (Fig. 8 *B2b*). However, no change was noticed in the precipitin lines for papain-digested type I myeloma protein (Fig. 8 *A2b* and *B2a*). By use of anti-normal γ-globulin absorbed with a mixture of type B Bence Jones proteins, the slow moving fraction of type I myeloma protein was completely removed (Fig. 8 *A3b* and *B3a*). However, no change was noticed in the papain-digested type II myeloma globulin (Fig. 8 *B3b*). After successive absorption of anti-normal γ-globulin serum with types A and B Bence Jones proteins, the slow moving component completely disappeared in both type I and type II myeloma globulins (Fig. 8 *A4b*, *B4a*, and *B4b*). It may be concluded that the S fraction of type I myeloma globulin is antigenically related to Bence Jones protein of type B. On the other hand, the S component of type II myeloma globulins has antigenic determinants in common with Bence Jones protein type A. These conclusions coincide with the following observations: myeloma patients having type I myeloma globulin usually excrete type B Bence Jones protein (*e.g.* Ag., Bi., and Ko.), and myeloma patients having type II myeloma globulin usually excrete type A Bence Jones protein (*e.g.* Ha., Pe., Ki., and Po.). No patient was found to have the combination of myeloma protein type I and Bence Jones protein type A or myeloma protein type II and Bence Jones protein type B.

The antigenic character of the F fragment of normal γ-globulin and gamma type myeloma globulins was also confirmed by use of anti-normal γ-globulin absorbed with the F fragment of papain-digested normal γ-globulin isolated by starch block electrophoresis. The F fragment is a specific part of the 7S γ-globulins, that is, it is missing in β_{2A} (Fig. 8 *C1a*), β_{2M} (Fig. 8 *C1b*), and both types of Bence Jones proteins, but it is the part common to normal γ-globulin and types I and II myeloma globulins.

Antiserum to papain-digested normal γ-globulin produced almost the same precipitin lines as did antiserum to the undigested protein (Compare the photos of Fig. 8 *1* and *7*). One of the papain-digested type I myeloma globulins (Si.) produced a prolonged F line with antiserum to the digested normal γ-globulin (Fig. 8 *B7a*). Occasional fast lines of this type in immunoelectrophoresis seemed to correspond with the fastest bands in starch gel electrophoresis.

Further identification of the Bence Jones proteins with the S fragment of papain-digested myeloma globulins was carried out as follows: The S fragments of normal γ-globulin and myeloma globulins of type I (Ag.) and type II (Po.) were separated by starch block electrophoresis and subjected to the Ouchterlony immunodiffusion test with Bence Jones proteins. The S fragment of type II was indistinguishable from type A Bence Jones protein in the reaction with anti-normal γ-globulin; that is, it made a fused line with the type A protein but a double spur with type B protein and a single spur with the S fragment of papain-digested normal γ-globulin or that of type I protein.

The S fragment of type I was antigenically similar to type B protein but had

additional determinants as was indicated by a small spur between them. A single spur was observed between S of type I and S of type II instead of the double spur that was expected from the absorption experiment (Fig. 9, right). On the other hand, the S of type I was quite like the S of normal γ-globulin as indicated by a fused precipitin line.

Specific antisera to type I myeloma globulin (Wi.) and to the S fragment of type II myeloma globulin (Po.) were also used for further identification of the relationships of these proteins. Antiserum to the type I myeloma protein reacted strongly with type B and weakly with type A Bence Jones proteins. Antiserum to the S of type II (Po.) was quite specific showing no cross-reaction with the other protein fragments prepared with papain (Fig. 8 *B9b*), whereas undigested normal γ-globulin and type II myeloma globulin (Ha.) produced a faint precipitin line with this antiserum. This phenomenon is like that encountered with antiserum to type A Bence Jones proteins. However, not only type B Bence Jones proteins but even type A failed to make any precipitin line with the antiserum to the S fragment of this type II protein in both the Ouchterlony and the quantitative precipitin tests. Only slight inhibition of the precipitate in the Ouchterlony test was found for the homologous system after absorption with type A Bence Jones protein.

5. Antigenic Relationships between β_{2A} Myeloma Globulins and the Two Types of Bence Jones Proteins.—Four samples of the β_{2A} type were identified by immunoelectrophoresis with horse anti-human serum. As shown in Figs. 2 and 3, the papain-digested β_{2A} myeloma globulin produced only one precipitin line in immunoelectrophoresis and a broad main band in starch gel electrophoresis. With specific antiserum (anti-type B Bence Jones protein (Lo.) or anti-macroglobulin digest (ND)) this precipitin line appeared for β_{2A} as it did with anti-normal γ-globulin (Fig. 8 *C1a* and *C6a*), but anti-normal γ-globulin absorbed with a mixture of type B Bence Jones proteins did not react with papain-digested β_{2A} myeloma globulin (Fig. 8 *C3a*). Though the electrophoretic position of the precipitin line produced by papain-digested β_{2A} myeloma globulin was similar to that of the F fragment of papain-digested normal γ-globulin, the absorption of anti-normal γ-globulin with F fragment did not affect the precipitin line of papain-digested β_{2A} myeloma globulin (Fig. 8 *C5a*). Specific antiserum to β_{2A} myeloma globulin (Ln.) produced an intense precipitin line with both the original and the papain-digested β_{2A} myeloma globulin (Fig. 8 *C8a*) and with other samples of β_{2A} myeloma globulins. Cross-reaction of the β_{2A} antiserum with papain-digested types I, II, and β_{2M}-globulins and with both types of Bence Jones proteins was faint. After the absorption of antiserum to β_{2A} myeloma protein with normal γ-globulin, homologous β_{2A} myeloma globulins still reacted with the absorbed and heterologous antiserum. These results suggest that β_{2A} myeloma globulins have at least two antigenic units; one is in common with type B Bence Jones protein and normal γ-globulin and another

is specific to β_{2A} myeloma globulin. Actually, four cases of β_{2A} type myeloma (Ln., Mo., No., and Sil.) all excreted type B Bence Jones protein in the urine, and no β_{2A} type myeloma patients were found to excrete type A Bence Jones protein.

6. Antigenic Relationships between β_{2M}-Globulin and the Two Types of Bence Jones Proteins.—As shown in Fig. 8 *C6b*, a papain-digested pathologic β_{2M}-globulin (Na.) produced two precipitin lines with the corresponding antiserum to the digest (anti-ND). The precipitin line having the faster mobility had antigenic determinants in common with type B Bence Jones protein according to the procedure in which specific antiserum against type B Bence Jones protein is used and also as shown by an absorption experiment with type B Bence Jones protein (the Osserman method). As was indicated by the unchanged pattern after an extensive absorption experiment, the slow moving fraction of the digested macroglobulin seemed to be the specific antigenic part of the macroglobulin. The antigenic character of β_{2M}-globulin will be reported in detail in another paper (26).

7. Micromolecular Myeloma Proteins and the Two Types of Bence Jones Proteins.—Five cases of Bence Jones proteinemia (serum micromolecular proteins) were studied. Four cases excreted type A Bence Jones protein, and the other case excreted type B Bence Jones protein. No difference was observed between the Bence Jones protein in the serum and the protein in the urine by electrophoresis, ultracentrifugation, or immunodiffusion.

In two cases of type II myeloma patients two precipitin lines of abnormal serum components were observed by immunoelectrophoresis with anti-normal γ-globulin. One line was identified as type II myeloma globulin by the Ouchterlony test on a sample purified by starch zone electrophoresis. Another line having the same mobility as the urinary Bence Jones protein of the same patient was identified as Bence Jones protein by the Ouchterlony test with anti-normal γ-globulin and with specific antiserum to type A Bence Jones protein. In one case examined by paper electrophoresis and ultracentrifugation, two peaks were also observed.

8. Antigenic Map of Normal γ-Globulin, Myeloma Globulins, and Bence Jones Proteins.—From the above results an antigenic "map" of γ-globulin and of related proteins was drawn schematically. As shown in Fig. 10, common antigenic determinants are found in the slow moving fragment of normal γ-globulin, type I myeloma protein, β_{2A} myeloma protein, the macroglobulin, and type B Bence Jones protein. A second antigenic fragment was found to be common to the slow moving fragment of normal γ-globulin and of type II myeloma globulin, and also to type A Bence Jones protein. However, these common antigenic parts are not exactly the same in all proteins; for example, a double spur is produced between the type B Bence Jones protein (Lo.) and normal γ-globulin with a mixture of antisera against normal γ-globulin and

the Lo. Bence Jones protein. This suggests that the pathologic γ-globulin–related protein has specific antigenic determinants of its own. With antiserum to normal γ-globulin, normal γ-globulin made a single big spur with Lo. Bence Jones protein, so it is difficult to conclude whether the characteristic determinants of the pathologic globulin are specific in the qualitative or quantitative sense. In further experiments the heat precipitation reaction for Bence Jones protein was carried out on papain-digested myeloma globulin fractions. In some cases, the slow moving fraction of type I myeloma globulins and of type

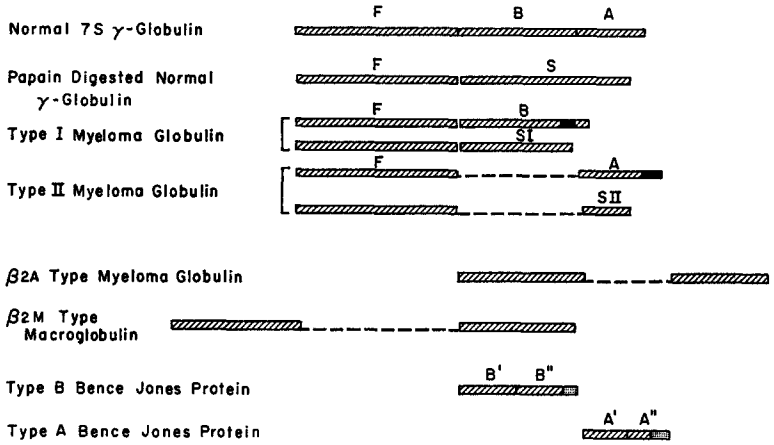

FIG. 10. Schematic map of the antigenic constitution of normal human 7S γ-globulin and its papain digest, types I and II myeloma 7S γ-globulins, β_{2A} myeloma globulin, β_{2M} macroglobulin, and types A and B Bence Jones proteins. *F* and *S* refer to the fast or slow moving papain-split fragments of normal γ-globulin. *A* and *B* denote portions antigenically similar to Bence Jones proteins of type A and B, respectively. Solid or dotted areas indicate parts antigenically different from normal γ-globulin.

II myeloma globulins (the part common to β_{2A}-and β_{2M}-globulins and to Bence Jones proteins) showed a positive heat precipitation reaction though the reaction was weaker than that of Bence Jones proteins. Although these experiments are incomplete, they support the hypothetical antigenic map of Fig. 10.

DISCUSSION

The structural significance of the multiple starch gel bands in the papain digests of 7S γ- and β_{2M}-globulins remains to be elucidated. It does not appear to reflect polymerization, for all the papain digests and those isolated fractions studied had an s_{20} of about 3.5S. One possibility is that the original globulins are electrochemically heterogeneous and that each F or S band represents the corresponding moiety of one of the original components. Alternatively, the bands may reflect progressive proteolytic removal of small peptides by papain to yield different cleavage products as occurs in chymotrypsinogen activation.

Since the multiple bands of the F fragment of a given protein are antigenically similar, and likewise for the S fragment, the current work is primarily concerned with the interpretation of the antigenic interrelationships of the immunoglobulins, myeloma globulins, macroglobulins, and Bence Jones proteins. Though the subject of several investigations (1, 2, 13–15) these interrelationships have been more clearly defined herein through comparison in many cases of the immunological type of myeloma globulin, its papain cleavage products, and the antigenic type of Bence Jones protein. For this purpose several homogeneous Bence Jones proteins selected from about 39 samples were used as standards for comparison with papain-split fragments of the myeloma globulins. The absorption tests in immunoelectrophoresis (Fig. 8) clearly indicated the close relationship between the S fragment of type I globulin and type B Bence Jones protein and between the S fragment of type II globulin and type A Bence Jones protein. By contrast, the S fragment of normal γ-globulin has a wider antigenic spectrum with determinants in common with both types A and B Bence Jones proteins. These facts serve to explain the Ouchterlony pattern of Fig. 9, in which normal γ-globulin made a single spur with type I or type II globulin but a double spur was observed between type I and type II myeloma globulins.

Concerning the molecular weight of these proteins, the S fragment of normal γ-globulin, the S of types I and II globulins, and most Bence Jones proteins have an $s_{20,w}$ of 3.5S. The estimated molecular weight of the S fragment of normal γ-globulin (14) is 40,000 to 55,000—similar to that of most Bence Jones proteins. Since the S fragments and Bence Jones proteins have about the same molecular size, a question arises about the meaning of the broader antigenicity of the S fragment of normal γ-globulin and the narrower antigenicity of the S fragment of type II myeloma globulin. Similar observations were made by Heremans (29) who found that the type II proteins are deficient in S antigens and give a faint precipitin line or sometimes none at all with anti-normal γ-globulin after absorption with F. Our results likewise indicate that the S fragment of type II—and also type A Bence Jones protein which is antigenically related to it—have fewer antigenic determinants in common with the S of normal γ-globulin. The fact that normal γ-globulin produced a precipitin line with anti-type A protein and with anti-S of type II, but S of normal γ-globulin never made a precipitin line with these antisera suggests that some of the antigenic determinants in common with type A protein may be lost during the papain digestion. Or as another possibility, the presence of double peaks in the quantitative precipitin curves (Fig. 6) suggests that there are two antigenic parts in type A protein, one in common with normal γ-globulin and another specific to type A proteins. Concerning the S of normal γ-globulin, two hypotheses will be considered: (*a*) Some of the homogeneous Bence Jones proteins have an $s_{20,w}$ of 2.0S as shown in Table I. Fahey and Askonas (16) got evidence that papain cleavage of mouse Bence Jones proteins produced 2.0S

fragments from 3.5S proteins. This suggests that the usual Bence Jones protein having an $s_{20,w}$ of 3.5S may be a dimer that could be represented by AA or BB in antigenic constitution instead of types A and B, respectively. So the S of normal γ-globulin might be AB. (*b*) In connection with the second hypothesis it was noted that the S of normal γ-globulin produces a diffuse band in starch gel electrophoresis, whereas that of type I or II produces several distinct bands (Fig. 2). Since the mobility of the S fragment of normal γ-globulin covers a range including all the distinct bands in both S fragments and since the S fragment of type I has distinct bands that migrate more rapidly to the cathode than those of type II, the assumption was made that the S of normal γ-globulin may be a mixture of these and possibly more components. Starch gel immunoelectrophoresis demonstrated that the multiple bands of the S fragment of a type II globulin were antigenically indistinguishable when tested with either anti-normal γ-globulin or anti-S of type II globulin.

According to the second hypothesis mentioned above the antigenic determinants of type A Bence Jones proteins or fragment S of type II could be represented by $A' + A'' + A''' + \ldots$. The S of type I could be $B' + B'' + B''' + \ldots$ (A''') and those of normal γ-globulin could be $A' + A'' + A''' + \ldots . B' + B'' + B''' + \ldots$. Since all the Bence Jones proteins were found to be heterogeneous in starch gel electrophoresis but their multiple bands were antigenically indistinguishable by homologous antiserum, the second hypothesis or a combination of the first and second hypotheses appears more correct; for in the first hypothesis, the normal γ-globulin should be homogeneous.

In the present paper the antigenic map of γ-globulin related proteins presented by Heremans (1) has been extended. Though the polymorphism of Bence Jones proteins and the F and S papain-digested fragments, as well as the number of peptide chains and the three dimensional configuration, have all been ignored in our scheme, this antigenic map is supported both by peptide maps and by the localization of genetic factors in the immunoglobulins and the Bence Jones proteins.

Peptide maps ("fingerprints") of tryptic digests of Bence Jones proteins reveal that there are few if any overlapping peptides between the type A and B proteins except for a spot due to the amino acid, arginine (30). On the other hand, many overlapping spots were found between normal γ, type I myeloma globulin, Bence Jones protein type B, and papain-digested macroglobulin, all of which share SI determinants. Almost all of the peptides of one type B Bence Jones protein were present in the myeloma globulin of type I (from the same patient, Ag.), but the globulin had many additional peptides, which presumably reflect the F determinants. Furthermore, preliminary experiments (31) indicate that the peptide maps of the F and S components of a type II myeloma globulin (Po.) have very little overlap with each other or with that of a Bence Jones protein of type B, though both the F and S components share many peptides with normal γ-globulin.

After the present work was completed, the localization of genetic factors in different areas of γ-globulin related proteins was reported by Harboe *et al.* (32) and by Franklin (33). Their conclusions completely coincide with our antigenic map. Both Gm(a) and Gm(b) factors were located in the F fragment of normal 7S γ-globulins and in some 7S γ myeloma globulins. The β_{2A}- and 19S γ-globulins lack the Gm characters as well as the F fragment. Inv(a) and Inv(b) characters were demonstrated in certain Bence Jones proteins, 7S γ-globulins, β_{2A} myeloma globulins and 19S globulins, but the Inv characters were found only in the S fraction after papain cleavage of 7S γ-globulins. Furthermore, the Inv activity was present in most Bence Jones proteins of type B but not in type A (33). It would be interesting to ascertain if either Gm(a) or Gm(b) factors were restricted to either type I or II myeloma globulins.

Also, after this work was completed, Franklin (34) reported in a preliminary note that there are two antigenically distinguishable types of β_{2A} myeloma globulins. Furthermore, in analogy to our findings for myeloma γ-globulins each of the β_{2A} immunological types was related to one of the major immunologically defined types of Bence Jones proteins and each had an antigenically distinct counterpart in the normal β_{2A}-globulin fraction. Although the β_{2A}-globulins we have studied have not been antigenically related to type A Bence Jones protein, Franklin's results further validate the importance of using the proteins produced by patients with multiple myeloma as a system for antigenic study of normal γ-globulins. In future work the relationship of these antigenic units to the polypeptide chains should be investigated and also the genetic factors controlling their biosynthesis.

Concerning the biosynthesis of these proteins, it is of interest that urinary γ-globulin (35) resembling Bence Jones proteins and antigenically deficient γ-globulin (36) resembling type II myeloma globulin have been found in normal urine and plasma, respectively. This raises again the question whether the elevated serum globulin in multiple myeloma is one of the normal serum components that has been increased in the disease or if it is a newly synthesized abnormal component.

This antigenic study suggests that structural units of the γ-globulin molecule (possibly polypeptide chains) exist which correspond to the three sets of antigenic determinants represented by F, A, and B, where the latter denote determinants equivalent to the two types of Bence Jones proteins. The A and B moieties are found in the S fragment of normal γ-globulin. If either the A or B subunits are over produced, with or without corresponding synthesis of F, three phenomena may result: (*a*) excretion of Bence Jones protein A or B, (*b*) increase in myeloma globulin type I or II without Bence Jones proteinuria, or (*c*) both a myeloma type hyperglobulinemia and a Bence Jones proteinuria. If the B subunit is over produced with a corresponding synthesis of the antigenic subunit characteristic of β_{2A}-globulin, the β_{2A} type globulin may be made

in excess. In view of Franklin's report (34) we must survey more extensively the types of Bence Jones proteins produced by patients who make β_{2A} myeloma globulins.

Only a few cases of macroglobulinemia with accompanying Bence Jones protein excretion have been reported in the literature. Though there is no description of the antigenic type of the Bence Jones protein in these cases, our results suggest it may be a type B protein. However, the need for antigenic study of more cases is apparent. Further study should also be made of the antigenic and structural relationships as ascertained by the peptide map method for myeloma serum globulins and Bence Jones proteins with particular emphasis on the serum and urinary proteins from the same patient.

SUMMARY

By means of immunodiffusion and immunoelectrophoresis study has been made of antigenic relationships of Bence Jones proteins, and the three classes of normal and pathological immunoglobulins, 7S γ, β_{2A}, and β_{2M}. All thirty-nine Bence Jones proteins studied could be classified into either one of two distinct antigenic types, A or B. Both types are related to the immunoelectrophoretically slow (S) fragment of a papain digest of normal γ-globulin; B is related more closely than A, but neither has antigenic determinants in common with the fast (F) fragment. The 7S γ myeloma globulins were either immunological type I or II. The papain digests of these proteins produced the S and F precipitin lines in immunoelectrophoresis but multiple bands in starch gel electrophoresis, especially in the F region. The S fraction of type I myeloma globulins is antigenically similar to Bence Jones protein of type B, and the S component of type II myeloma globulins has antigenic determinants in common with type A Bence Jones protein. Correspondingly, myeloma patients with type I globulins and proteinuria usually excrete type B Bence Jones proteins, whereas patients with type II excrete type A proteins. The F fragment is the part common to normal 7S γ-globulin and types I and II myeloma globulins but is absent in β_{2A} and β_{2M} pathological globulins and in both types of Bence Jones proteins. Papain digests of β_{2A} myeloma globulins produced a single precipitin line in immunoelectrophoresis. β_{2A} myeloma globulins appeared to have two antigenic units, one in common with type B Bence Jones protein and normal γ-globulin, and another specific to β_{2A}. The β_{2A} myeloma patients excreted type B Bence Jones protein. The papain digest of a macroglobulin produced two precipitin lines, the faster of which had antigenic determinants in common with type B Bence Jones protein, the slower seemed specific for the macroglobulin. Five serum micromolecular globulins proved to be either type A or B Bence Jones proteins. From the above results, an antigenic map was constructed showing which determinants are shared and which are specific for normal 7S γ-globulin, types I and II myeloma globulins, β_{2A} myeloma globulins, a macroglobulin, and types A and B Bence Jones proteins.

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