Peptide Chromatograms of Normal Human and Myeloma γ-Globulins*

MELVIN FRIED AND FRANK W. PUTNAM

From the Department of Biochemistry, University of Florida College of Medicine, Gainesville, Florida

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The peptides resulting from the tryptic hydrolysis of four myeloma globulins showing the electrophoretic characteristics of γ-globulin have been compared with the peptides resulting from pooled normal human γ-globulin. Although some differences were found, the evidence suggests that large segments of the polypeptide structure of normal γ-globulin and of the myeloma globulins are identical, with the possibility of interchanges of amino acids occurring in the N-terminal position and at various other loci characteristic of the protein from individual patients.

The γ-globulins are considered to be a group of structurally related proteins with similar origin and function but exhibiting a continuous variation in biological, chemical, and physical properties (Fahey and Horbett, 1959). Thus, the γ-globulins are heterogeneous by whatever criterion studied, and despite repeated attempts it has not yet been possible to isolate a homogeneous component from the many individual species (Phelps and Putnam, 1960). This heterogeneity as well as their high molecular weight have hindered structural study. The globulins formed by patients with multiple myeloma offer an advantage in this respect because of their greater homogeneity (Putnam and Udin, 1953). Although often individually homogeneous, the myeloma globulins fall into several immunoelectrophoretic classes (*e.g.*, γ, β_{2A}, β_{2M}, etc.) and several molecular weight classes (*e.g.*, 7 *S*, 11–14 *S*, 19 *S*, etc.) (Putnam, 1957). In the interest of making a structural comparison of a group of closely related proteins this study was restricted to normal human γ-globulin and myeloma globulins of the 7 *S* γ-type. Although these proteins are very similar in antigenic and physical properties, significant differences in amino acid composition (Smith *et al.*, 1956) and N-terminal groups (Putnam, 1958, Sjöquist and Laurell, 1961) have been reported. Since the myeloma globulins are formed in the plasma cells (Meyer, 1962), which are a site of synthesis of immune γ-globulins (Putnam, 1957), the structural comparison of the normal and abnormal proteins is of interest for the general question of antibody structure.

In this study, a technique was employed which permits the comparison of the primary structures of the normal and myeloma globulins. It involved the ion exchange chromatography of the peptides obtained when the proteins were subjected to complete hydrolysis by trypsin. This procedure had already been applied to the study of the urinary Bence-Jones proteins (Fried and Putnam, 1960) which are also found in patients with multiple myeloma. The results of the present investigation indicate considerable structural similarity among the proteins studied, although characteristic individual differences are apparent.

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EXPERIMENTAL

Materials and Methods

γ-Globulins.—Prior to characterization, all of the proteins were isolated from serum by repeated salt precipitation and were then lyophilized. Table I lists some of the physical and chemical properties previously determined (Putnam, 1955) for the normal γ-globulin, a pooled sample (Merck, No. 45875), and the four myeloma globulins which were studied. The electrophoretic mobilities of the myeloma globulins place them in the γ range. Three of them were cryoglobulins, displaying relative insolubility in cold solutions. The s_{20} values cover a small range and are consistent with the molecular weights of 150,000 to 160,000 reported for γ-globulins. As determined by the Ouchterlony technique, all belonged to one antigenic group (Korngold and Lipari, 1956). The most striking differences are in their amino end-groups, which, as determined by Sanger's method (Sanger, 1945), vary in both number and kind. This indicates differences, not only in the type of N-terminal amino acid but possibly also in the ease with which these end-groups may react with dinitrofluorobenzene, a property which is probably related to the three-dimensional configuration of a protein if not to its primary structure (Porter and Press, 1962).

Hydrolysis by Trypsin.—The techniques described earlier (Fried and Putnam, 1960) were employed for the hydrolyses. The proteins were first dialyzed against *p*H 2.3 citrate buffer for 4 hours in the cold, followed by dialysis against cold-distilled water until all traces of buffer were gone. This results in the removal of most of the trichloroacetic acid–soluble, ninhydrin-reactive material sometimes found tightly adsorbed even

TABLE I
COMPARISON OF PHYSICAL PROPERTIES OF SERUM PROTEINS

Protein	*p*I	Mobility (*p*H 8.6)	$S_{(20)}$[a]	Antigenic Group	Amino End-Groups (moles/160,000 g)	
					Asp	*Glu*
Normal γ	7.2	−1.3	6.6		1.1	1.8
Gu[b]	[c]	[c]	6.0	I	2.3	2.2
Se[b]		−0.8	6.4	I	0	2.7
Th[b]	7.5	−1.1	6.6	I	1.8	(0.2)
Wi	6.6	−1.1	7.0	I	1.8	(0.1)

[a] Uncorrected for concentration. [b] Cryoglobulins. [c] Insoluble.

to crystalline proteins. The proteins were then oxidized with performic acid (Hirs, 1956). The resulting material was suspended in *p*H 7.8 buffer at concentrations of 3–5 mg per ml, and was subjected to the action of trypsin (Armour, twice recrystallized) at 37° for 48 hours, at a relative protein-to-enzyme concentration of 100:1. Toluene was added to inhibit microbial action. The reaction was followed by colorimetric ninhydrin determinations (Moore and Stein, 1954) and was terminated by bringing the hydrolysates to *p*H 2.2 and freezing. Hydrolysis was very rapid initially, reaching about 90% in 30 minutes, and was essentially complete in 24 hours. The addition of more trypsin after 24 hours did not increase the extent of hydrolysis. As noted earlier

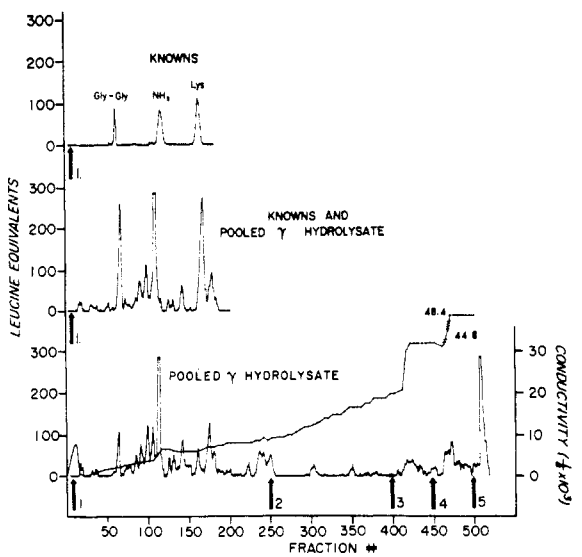

FIG. 1.—Chromatograms of known compounds, a γ-globulin hydrolysate, and a mixture of the knowns with the γ-globulin hydrolysate. The conductivity of the effluent fraction is plotted in parallel with the peptide chromatogram of the γ-globulin hydrolysate. The eluting solvents are as follows: 1. A gradient produced by running 0.5 N *p*H 5.1 buffer into a mixing chamber containing 500 ml of 0.2 N *p*H 3.3 buffer for 500 ml (250 fractions). 2. 1.0 N *p*H 5.1 buffer is introduced into the mixing chamber for 300 ml (150 fractions). 3. 1.0 N *p*H 5.1 buffer is placed directly on the column. 4. 2.0 N *p*H 5.1 buffer is placed directly on the column. 5. 2.0 N NaOH is placed directly on the column. Leucine equivalents in μmoles per liter.

TABLE II
DEGREE OF HYDROLYSIS OF PROTEINS

Protein	Increase in Leucine Equivalents per 100 moles of Basic Amino Acids
Normal γ	76
Gu	130
Se	80
Th	71
Wi	117

(Fried and Putnam, 1960), the tryptic digestion of performate-oxidized protein was more rapid and apparently more complete than that of non-oxidized material.

The completeness of hydrolysis was estimated by comparing the increase in ninhydrin equivalents during hydrolysis with the number of basic amino acids in the proteins derived from average values for γ-globulins (Brand, 1946; Smith *et al.*, 1956). Since trypsin hydrolyzes significantly only those peptide bonds in which the carboxyl group is donated by arginine or lysine, these two values should be approximately equal. As is shown in Table II, the increase in ninhydrin color equivalents varied from 70–130% of the calculated values. This is in good agreement considering the fact that the ninhydrin color values for the complex peptides resulting from hydrolysis are unknown and probably differ from those of simple compounds. Since significant differences in the basic amino acid composition of myeloma globulins have been reported even for those with similar electrophoretic mobility (Smith *et al.*, 1956), a variation in the number of bonds broken may reflect the composition rather than incomplete hydrolysis.

Chromatography.—Aliquots of hydrolysate equivalent to 15 mg of protein, as calculated from Kjeldahl N values, were chromatographed as described earlier (Fried and Putnam, 1960) by a modification of the method of Hirs *et al.* (1956). The 60 × 0.9 cm columns of Dowex 50 x 2 were eluted at 50° with a series of citrate buffers which resulted in an increasing *p*H and ionic strength gradient (Fig. 1). Fractions of 2 ml were collected automatically and analyzed manually by the photometric ninhydrin method.

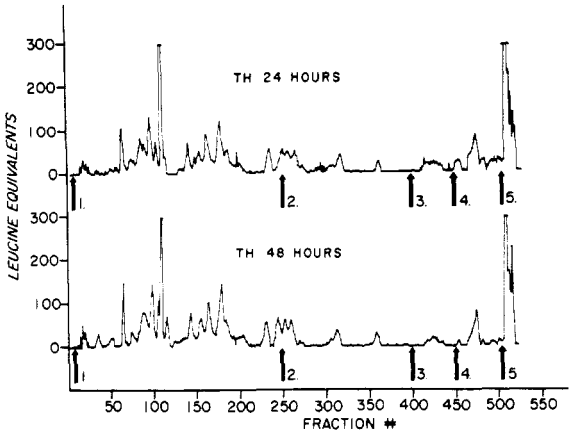

FIG. 2.—Comparisons of separate 24 and 48 hour hydrolysates of myeloma globulin TH. Eluting solvents as in Fig. 1. Leucine equivalents in μmoles per liter.

The chromatography proved to be quite reproducible. Although the positions of individual peaks might vary by several fractions, particularly with the age of the resin, the position of peaks relative to one another did not change from chromatogram to chromatogram.

In Figure 1 are shown the positions of some known compounds, and also a chromatogram in which these had been added to a hydrolysate of a normal γ-globulin. It is apparent that the chromatographic positions of these compounds were not changed by the hydrolysate, and that, conversely, the known compounds did not affect the positions of the peptides in the hydrolysate. The position of ammonia represented by the tall peak in fractions number 120–125 was verified by Conway analysis. Aliquots representing peaks at a number of positions were isolated and shown to contain peptides both by increase in ninhydrin color after acid hydrolysis and by analysis of the amino acid content with the Spinco amino acid analzyer. There was a qualitative similarity in the amino acid composition of peptides eluted at corresponding positions from the different proteins.[1]

RESULTS

The validity of comparisons of peptide patterns obtained from different proteins depends upon completeness of hydrolysis and reproducibility of the chromatographic separation. The first factor has been considered in the preceding section; the second is illustrated in Figure 2. This compares the chromatographic patterns of the peptides resulting from a 24-hour hydrolysis of one of the myeloma globulins (TH) with those from a 48-hour hydrolysis of the same protein. All

[1] The chromatographic characterization and quantitative analysis of peptides obtained by tryptic hydrolysis of normal γ-globulin and myeloma globulins is being undertaken.

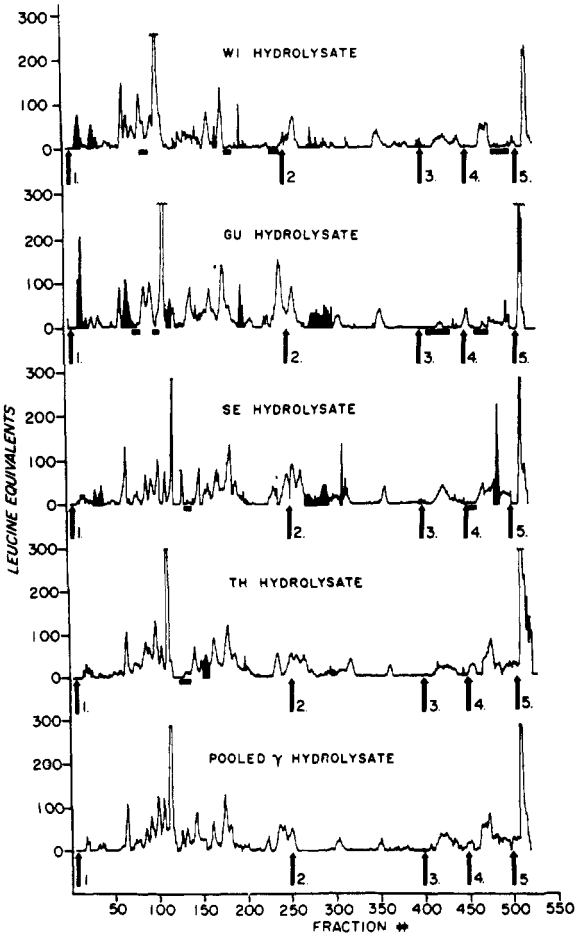

FIG. 3.—Chromatograms of tryptic hydrolysates of myeloma globulins and pooled γ-globulin. Solid peaks represent those present in the myeloma globulins but absent from the pooled γ-globulin. Areas in the chromatograms of the myeloma globulin from which peaks present in the γ-globulin peptide chromatogram are absent are underlined. Eluting solvents as in Fig. 1. Leucine equivalents in μmoles per liter.

preparative steps, including oxidation of the protein, hydrolysis, and separation of the resulting peptides, were performed separately. With the exception of slightly better resolution in the chromatogram of the 24-hour hydrolysate, the chromatograms are nearly identical. It is also apparent from Figure 2 and from Figure 3 that a finite number of peaks representing peptides results from the hydrolysis of the pooled normal γ-globulin as well as the myeloma proteins.

The chromatograms of the myeloma proteins in Figure 3 have been arranged in the order of increasing similarity to the normal γ-globulin. This order is in no way paralleled by relationships in the properties listed in Table I. Comparisons between any two abnormal proteins or between any myeloma globulin and the pooled normal sample reveal at most six or eight discrepancies in the patterns. A large majority of the peptide

peaks appear in the same positions and in relatively the same quantities in all of the chromatograms. This constancy among the patterns is particularly marked in the seven well-defined peaks appearing from fractions number 60 to 120, the five or six peaks at about fractions 130 to 180, and the four peaks usually observed in the range 220 to 270. It should also be noted, however, that, despite the manifold similarities, in every case there are peptides present in one chromatogram which are not present in the others. These comparisons are of course restricted to those peptides which were separated prior to the addition of NaOH. There appears to be some degree of correlation between new peaks and "missing" peaks in the myeloma patterns. For example, in the TH hydrolysate there is one new peak and one missing peak. However, a quantitative relationship between new peaks and missing peaks was not found in all instances.

Discussion

In the literature on γ-globulins, one encounters evidence both for a considerable diversity and for gross similarity in their structure. Most earlier methods applied to normal and pathologic γ-globulins reflected the molecules as a whole. Even in amino acid analysis the high molecular weight of γ-globulin tends to obscure differences because of the large number of residues of each type. N-terminal group determinations have demonstrated that structural differences exist among normal and pathological γ-globulins (Putnam, 1955, 1958; Sjöquist and Laurell, 1961). Yet, except for a single pentapeptide sequence in rabbit γ-globulin (Porter, 1950), there is no information about the amino acid sequence of any of the γ-globulins. The procedures used in this investigation were designed to reflect the structural moieties of which these molecules are composed. Widespread dissimilarities in amino acid sequence should have resulted in the formation of totally dissimilar peptides, and correspondingly divergent chromatograms.

The observation of a limited number of peptide peaks, especially in the pooled human γ-globulin, is significant in view of the concept of normal γ-globulin as representing the summation of a continuous spectrum of molecular species. If there were widespread continuous variations in the primary structures of this group of protein entities, it might be expected that a smear of peptides would result from the procedures used, rather than the observed series of discrete peaks. Also, since there are between 120 and 135 arginyl and lysyl residues per mole of γ-globulin, a similar number of peptides is to be anticipated from the complete hydrolysis of such a protein. However, in all of the figures, only 30 to 35 peaks are visible. Undoubtedly some of the peaks represent more than one peptide, but the small number of peptide peaks is still remarkable. One explanation for this finding would be that the protein is made up of several fairly large subunits, which tend to duplicate among themselves the peptide primary structure. This suggestion is supported by the findings of Porter (1959), Hsiao and Putnam (1961), and others. These workers found that γ-globulins are rather easily split into chromatographically different but electrophoretically and ultracentrifugally similar subunits by the action of papain and other proteolytic enzymes.

The observed over-all similarities in the chromatograms, which were based on procedures that yielded reproducible results, can only mean that normal and abnormal γ-globulins have many sequences in common. The structural differences suggested by the peptide chromatograms probably represent changes in short interior sequences as well as the variations in the end-group already noted.[2]

It thus appears that in response to unknown stimuli the plasma cells present in the tumors of myeloma patients produce globulins that are structurally similar to normal γ-globulin over large portions of the polypeptide chains, although interchanges of amino acids may occur at various loci, characteristic of the protein from individual patients. This conclusion is in contrast to our previous finding that the Bence-Jones proteins excreted by myeloma patients exhibit a gross dissimilarity by the same criterion (Fried and Putnam, 1960).

Considered more broadly, these data have several implications. The first is that the γ-globulins of any individual or any closely related group of individuals most probably have the same basic primary structure accompanied by minor though characteristic modifications. Secondly, when disease processes elicit changes in the production of γ-globulin, the new proteins produced must reflect the genetic makeup of the clone of cells in which they are synthesized in respect both to a primary common pattern and to characteristic structural alterations. Finally, from the studies of normal γ-globulin and myeloma γ-globulins, it should be anticipated that antibody γ-globulins will be found to possess the same underlying structure and that the differences which endow antibodies with their specificity will be found to be ascribable to no more than small, narrowly-localized sequences of amino acids.

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[2] The general conclusions reported herein have been verified by peptide patterns obtained with two-dimensional paper chromatography and electrophoresis (Putnam, 1962)

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Optical Rotatory Dispersion of G-Actin

BÉLA NAGY AND WILLIAM P. JENCKS

*From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts**

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Optical rotatory dispersion data for G-actin prepared by several different methods are presented. Reproducible values of $[\alpha]_D = -44°$, $\lambda_c = 252$ mμ, and $b_0 = -184$ were obtained for purified preparations obtained by cold-extraction or by repeated polymerization and depolymerization, including a Mg^{++}-induced polymerization step. Actin which had been extracted at room temperature or subjected to only a single Mg^{++}-induced polymerization step gave rotatory dispersion values suggesting the presence of a protein other than native G-actin-ATP. The presence of a protein with the properties of tropomyosin in preparations which had been extracted at room temperature was confirmed. From the change in optical rotatory properties and the increased susceptibility to proteolytic digestion by a bacterial protease, by chymotrypsin, and by trypsin after the loss of bound nucleotide, it is concluded that the loss of the bound nucleotide causes a change in the protein conformation. Optical rotatory dispersion reveals three structural states: the most ordered state in the presence of the bound nucleotide ($b_0 = -184$), a less ordered state after the bound nucleotide is removed ($b_0 = -74$), and a random coil found in concentrated urea or guanidine-HCl solution ($b_0 = 0$).

The muscle protein, actin, has a tightly bound adenine nucleotide (Straub and Feuer, 1950; Laki *et al.*, 1950; Mommaerts, 1952b), which undergoes dephosphorylation during polymerization and a rephosphorylation (Strohman, 1959), or, more likely an exchange reaction (Martonosi *et al.*, 1960) upon depolymerization *in vitro*. The removal of the bound nucleotide from actin is an irreversible process under most conditions, although under certain conditions a time-dependent partial reversibility has been observed (Bárány *et al.*, 1961a,b; Grubhofer and Weber, 1961; Maruyama and Gergely, 1961; Strohman and Samorodin, 1962). It has been suggested that the dissociation of G-actin and ATP is accompanied by a conformational change of the protein (Asakura, 1961; Strohman and Samorodin, 1962).

This study concerns the optical rotatory properties of actin with and without its bound nucleotide. Some optical rotatory dispersion data on actin have been published by Kay (1960) and some unpublished observations are mentioned elsewhere (Asakura, 1961; Laki and Kenton, 1961). Data will be presented which indicate that Kay's observation represents the special case of a nucleotide-free actin. Data will also be given showing that changes in optical rotatory parameters and in the susceptibility to proteolytic digestion indicate changes in the conformation of actin when the bound nucleotide is lost.

MATERIALS AND METHODS

Three different methods of actin preparation were used. Crude actin was prepared according to Bárány and Bárány (1959). The acetone-dried powder was extracted with 25 volumes of H_2O for 20 minutes at room temperature (warm extract) or at 4° (cold extract, Drabikowski and Gergely, 1962) and purified by ultracentrifugation according to the method of Mommaerts (1951).

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