Structural Differences between Macroglobulins Belonging to Two Serologically Distinguishable Subclasses*

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ABSTRACT: Peptide maps were prepared from μ chains and native proteins of 25 macroglobulins belonging to two antigenically distinguishable subclasses. The over-all appearance of the peptide maps of all the heavy chains was similar in that about 75% of the peptides were the same in all and 25% differed. The two serologically distinguishable subclasses differed from each other in only a small number of peptides. All of the reactive proteins had a single or double peptide which was lacking in all the nonreactive proteins. Three of the nonreactive proteins differed from the remainder in having a different group of peptides in the same area of the peptide map. The peptide characteristic of the reactive proteins was located in the Fab fragments prepared from two of the proteins. The over-all structural arrangement of a constant and a variable region in the μ chains appears to be similar to that of the light chains and γ chains. The significance of the structural differences among the subclasses in relation to possible genetic polymorphisms remains to be determined.

Previous studies (Frangione and Franklin 1965; Putnam *et al.*, 1967) have demonstrated that peptide maps of μ chains differ from those of the γ chains, thus suggesting that the two types of heavy chains have different amino acid sequences. This interpretation is consistent with the results of amino acid analyses of several γ and μ chains (Heimer *et al*; 1962; Press and Piggot, 1967; Chaplin *et al.*, 1965). Comparison of peptide maps of μ chains prepared from different macroglobulins showed the existence of a number of peptides common to all of them and others unique to each (Frangione and Franklin, 1965; Putnam *et al.*, 1967). Since amino acid sequence studies of several light chains (Hilschmann and Craig, 1965; Putnam *et al.*, 1966; Milstein, 1966a,b) have demonstrated a region common to all of the proteins belonging to a given class and another stretch which is unique for each protein, and because a similar over-all structure has been postulated for the γ chains (Frangione *et al.*, 1966, 1967; Hill *et al.*, 1966; Milstein, 1966a,b; Porter, 1967), it seems likely that the μ chains also consist of a constant and a variable region. The present report provides additional data consistent with this view and localizes most of these differences to the amino-terminal half of the molecule.

As a result of intensive immunologic studies in recent years, differences between heavy chains of paraproteins belonging to each of the three major classes of immunoglobulins have been noted, and in the case of the γ, α, and μ chains, it has been possible to classify these differences so as to define subclasses (Grey and Kunkel, 1964; Terry and Fahey, 1964; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966; Feinstein *et al.*, 1966; Harboe *et al.*, 1965; Franklin and Frangione, 1967). γ chains have been divided into four types: γ1, γ2, γ3, and γ4. Peptide maps (Frangione *et al.*, 1966) and sequence studies at the carboxy-terminal end (Porter, 1967) and the region of the intrachain disulfide bridges of the Fc fragment (Milstein, 1966a,b) have revealed only a small number of differences in the primary structure of Fc fragments belonging to the γ-chain subclasses. In the case of the two types of α chains, some differences in the heavy–light disulfide bonds have been noted (Grey, 1968). With the recognition of at least two antigenically distinguishable subclasses of μ chains (Franklin and Frangione, 1967), it seemed desirable to determine if they differ in their primary structure. To this end, peptide maps were prepared from the μ chains of 25 macroglobulins, 15 of which belonged to the major nonreactive group and ten to the minor reactive one.[1] Comparison of these peptide maps showed at least three, and possibly four, distinct patterns differing in only a few peptides. Two of these patterns were associated with the major nonreactive antigenic type while the others corresponded to the minor group of reactive proteins.

Materials and Methods

Macroglobulins were isolated from the sera of 25 patients with macroglobulinemia of Waldenstrom. Most of these were the same as those described previously (Franklin and Frangione, 1967). Seven proteins were cryoglobulins and were isolated by repeated precipita-

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[1] Minor and major are arbitrarily used to designate the two groups on the basis of their frequency among 25 macroglobulins studied. The terms μ1 and μ2 (*Bull. World Health Organ. 30,* 447 (1964)) have not been introduced since a larger number of proteins will have to be studied to definitively determine their exact frequency. The antiserum is the same as that described in a previous publication (Franklin and Frangione, 1967).

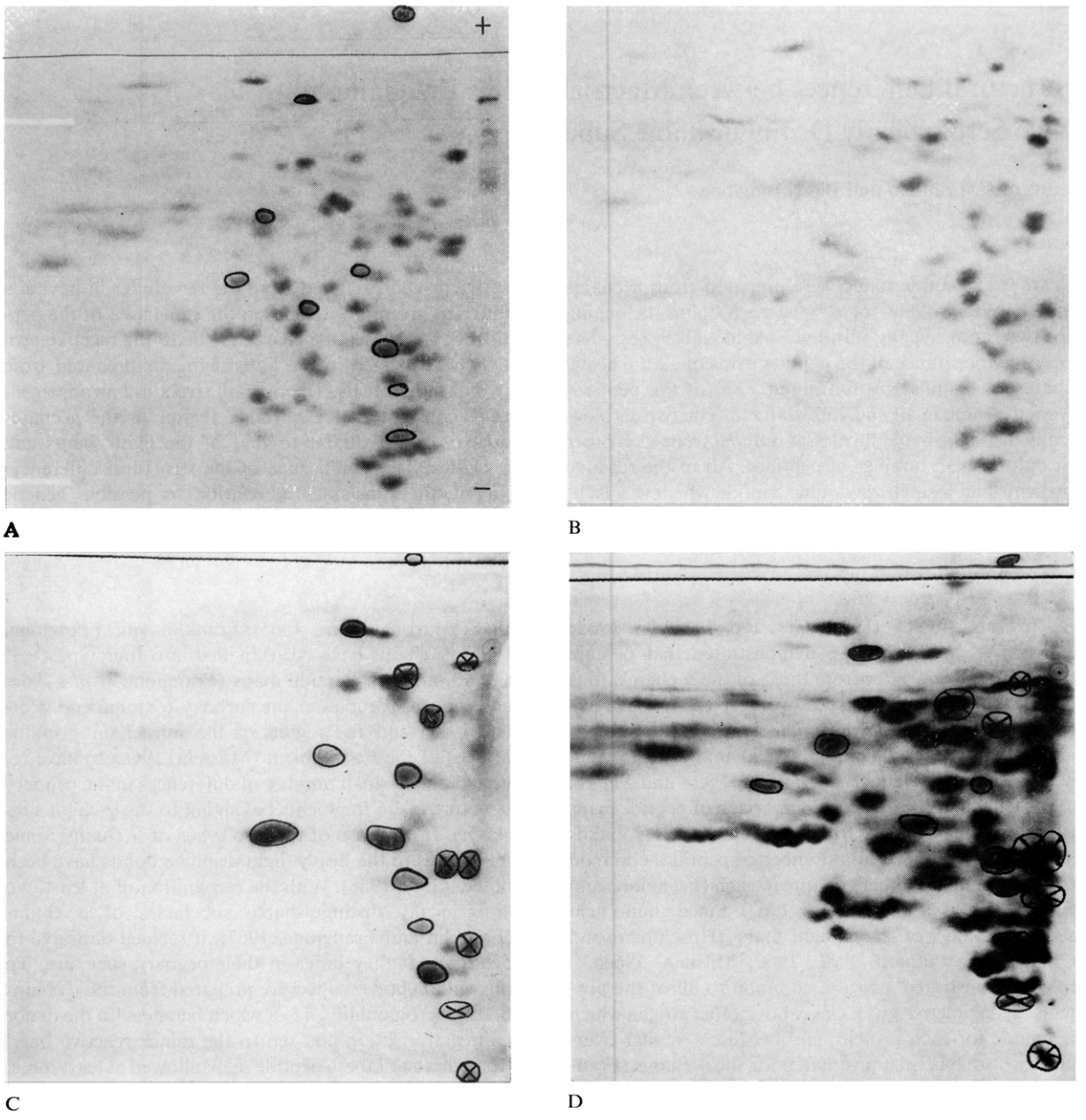


FIGURE 1: Peptide maps of trypsin digests of (A) macroglobulin 6, (B) μ chain, (C) light chain, and (D) a 2:1 mixture by weight of μ and light chains. Only a few light-chain peptides are visible in the native protein (A). However, most of these are restored by doubling the concentration of the homologous light chain. ○ indicates light-chain peptides. ⊗ indicates those which appear only after the addition of additional light chains (D).

tion at 0–4° followed by resolution at 37° (Franklin and Frangione, 1967). Fourteen were water-insoluble euglobulins which were partially purified by repeated dialysis against distilled water, followed by resolution in 0.3 M saline. The remaining four proteins, which were soluble in distilled water, were first subjected to starch zone electrophoresis (Kunkel and Slater, 1952). The homogeneous protein peak eluted from the starch block was then concentrated by vacuum dialysis. Final purification of the euglobulins and the proteins isolated by starch zone electrophoresis was accomplished by filtration through columns (100 × 3 cm) containing Sephadex G-200 in 0.3 M NaCl. The initial peak in all cases was more than 95% pure γM-globulin, as determined by immunologic assays for γ, α, and μ chains and κ and λ chains (Frangione and Franklin, 1965).

Identification of the two subclasses of μ chains was performed with a single antiserum (III) prepared in a rabbit to a macroglobulin (12) belonging to the minor reactive type and absorbed with umbilical cord serum and macroglobulin 13 belonging to the major nonreactive type (Franklin and Frangione, 1967). To date it has not been possible to prepare an antiserum specific for the major nonreactive subgroup, and consequently,

proteins belonging to this group have had to be identified by their failure to react with the antiserum specific for the minor reactive group.

Heavy- and light-polypeptide chains were prepared by the method of Fleischman (Fleischman *et al.*, 1963), using 0.75 M mercaptoethanol. The heavy and light chains were separated by chromatography on Sephadex G-100 in 1 M proprionic acid. Purity was generally tested with antisera to κ, λ, and μ chains. The light chains were immunologically pure while the μ chains were occasionally contaminated with approximately 2–5% of light chains. Control experiments with isolated light chains and light chains mixed with immunologically pure heavy chains indicated that amounts significantly in excess of 10% would be necessary to give rise to peptides derived from the light chains in peptide maps prepared from heavy chains.

The technique of peptide mapping was similar to that described previously (Frangione *et al.*, 1966). All proteins were oxidized with performic acid prior to trypsinization. Descending chromatography was performed for 20 hr as the first step, using butanol–acetic acid–water (4:1:5) as the solvent. This was followed by high-voltage electrophoresis in a pH 3.8 pyridine–acetic acid–water buffer (1:10:289) in a direction perpendicular to the chromatographic separation. All papers were dipped in ninhydrin and photographed. Peptide maps were compared on the photographs rather than the original paper patterns. Arginine and histidine were assayed by the methods of Sakaguchi and Pauly, respectively (Bailey, 1962).

Fragments lacking most of the μ-chain determinants, which probably corresponded to the amino-terminal half of the molecule (Fab^1 or $F(ab^1)_2$), were prepared by digestion with pepsin in pH 4 acetate buffer (0.082 M HAc–0.018 M NaAc) using 3 mg of enzyme/100 mg of protein (Kishimoto *et al.*, 1968). Following dialysis against 0.15 M NaCl, about one-third to two-thirds of the protein was lost as dialyzable peptides and the residual protein reacted only weakly or not at all with antisera to μ chains. In three instances, this material was further purified by passage through Sephadex G-75. Only the first peak was used for further studies. In studies where both the Fab and the Fc fragment were used, papain digestion, according to the method of Mihaesco and Seligmann (Mihaesco and Seligmann, 1968), was employed. Four macroglobulins, two of each class, were dissolved in pH 7 buffered saline (0.075 M NaCl–0.046 M Na_2HPO_4–0.029 M NaH_2PO_4) at a concentration of 10 mg/ml and were made 0.001 M in cysteine hydrochloride and 0.001 M in EDTA. The protein was digested with papain (2 mg/100 mg of protein) at 37° for 24 hr. The reaction was stopped by the addition of 0.002 M iodoacetamide. After the addition of salt to make the solution 1 M, it was immediately applied to a Sephadex G-200 column previously equilibrated with pH 8.2 Tris buffer–1 M NaCl. As described (Mihaesco and Seligmann, 1968), the initial peak, which made up less than 10% of the total, reacted only with the antiserum to μ chains and not with antisera to the Fab fragment of γ-globulin or light chains, while the second peak reacted only with the antiserum to Fab fragment. The third peak failed to react with either of the antisera and consisted of small peptides.

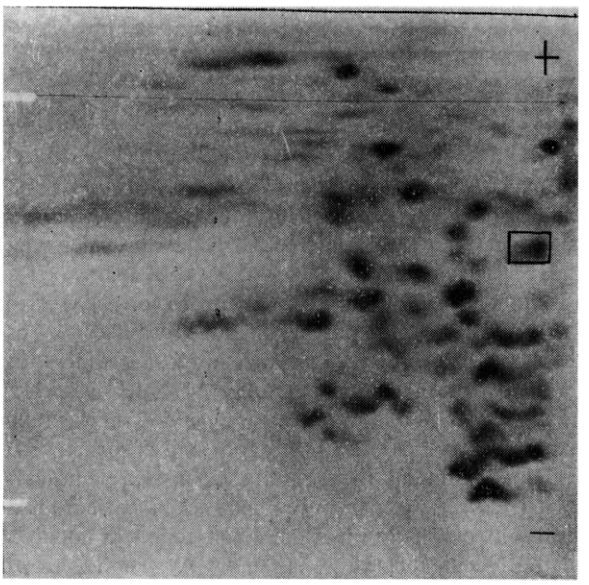
Results

Comparison of μ Chains–Light Chains–Native Proteins and Pepsin Digests. The μ chains prepared by the method described were essentially free of light chains since they either failed to react with antisera to light chains or reacted only weakly when tested at concentrations greater than 10 mg/ml. Comparison of the μ chains of 25 of these proteins to each other and, in most instances, to the native proteins demonstrated several significant points. Firstly, in all instances, the peptide maps of the native proteins resembled those of the μ chains closely. In 21 of 25 proteins, the maps of the native proteins contained less than ten peptides that could be identified clearly as originating from the light chains in addition to the μ-chain peptides (Figure 1A–C). Peptide maps of only four of the native proteins contained a significant number (ten or more) of peptides not present in the μ chains prepared from them. Peptide maps of the trypsin digests of artificial mixtures of heavy and light chain in a proportion of 2:1 by weight demonstrated the presence of most of these missing light chain peptides (Figure 1D). Secondly, comparison of the peptide maps of the peptide maps of the μ chains from each of these 25 proteins demonstrated a striking similarity in their appearance (Figure 2A–D). There were from 45 to 50 peptides in the maps of the μ chains of which 25–30 were darkly stained. While the majority of these were the same for all of the proteins, each μ-chain map had approximately five to eight peptides whose combined appearance served to distinguish it from the other μ-chain fingerprints. This result is consistent with findings previously reported for γ chains (Frangione *et al.*, 1967). The small number of unique peptides noted suggests that the variable region of the μ chain is rather limited, and that it may include only a small portion of the heavy chain.

To more clearly delineate the number of constant and variable peptides and to localize the variable region more precisely, peptide maps were prepared from the pepsin digests of nine of the macroglobulins. In three instances, peptide maps were prepared from the total nondialyzable residue after pepsin digestions and also from the first peak eluted from Sephadex G-75. Since the maps from both products were identical, the remaining six proteins were not subjected to Sephadex filtration after dialysis.

Following pepsin digestion, the recovery of protein ranged from 40 to 60%. The material reacted strongly with an antiserum to the Fab fragment of γG-globulin and only weakly, if at all, with an antiserum to the μ chain. On the basis of these findings and previous studies by others (Kishomoto *et al.*, 1968), it seems likely that this product consists of the light chain and the amino-terminal half of the heavy chain, and that it is analogous to the Fab fragment of γG-globulin.

Comparison of the peptide maps of the μ chains, the light chains, and the pepsin digests of each of nine macroglobulins permitted the identification of peptides de-



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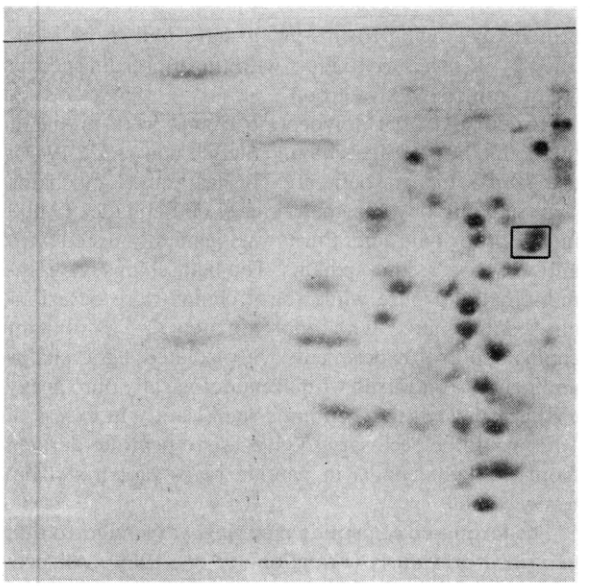

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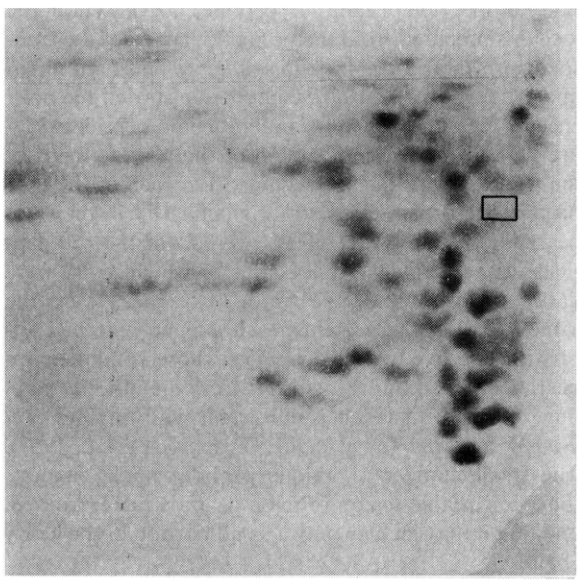

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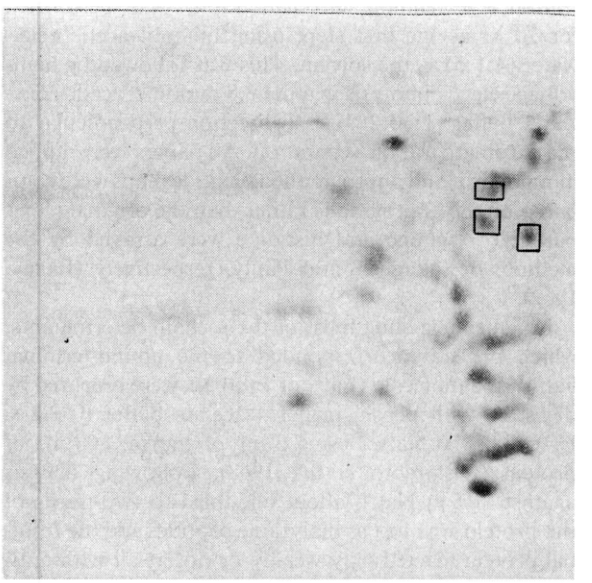

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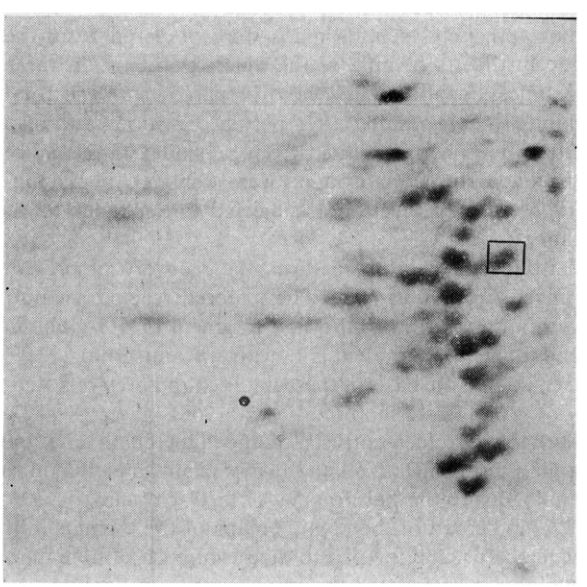

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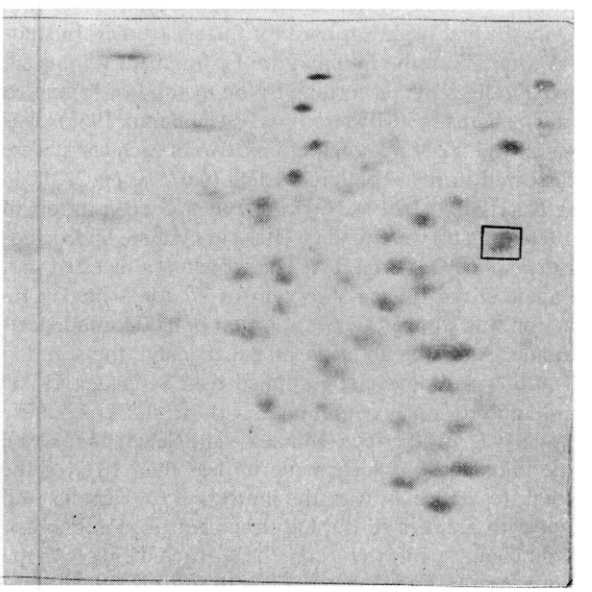

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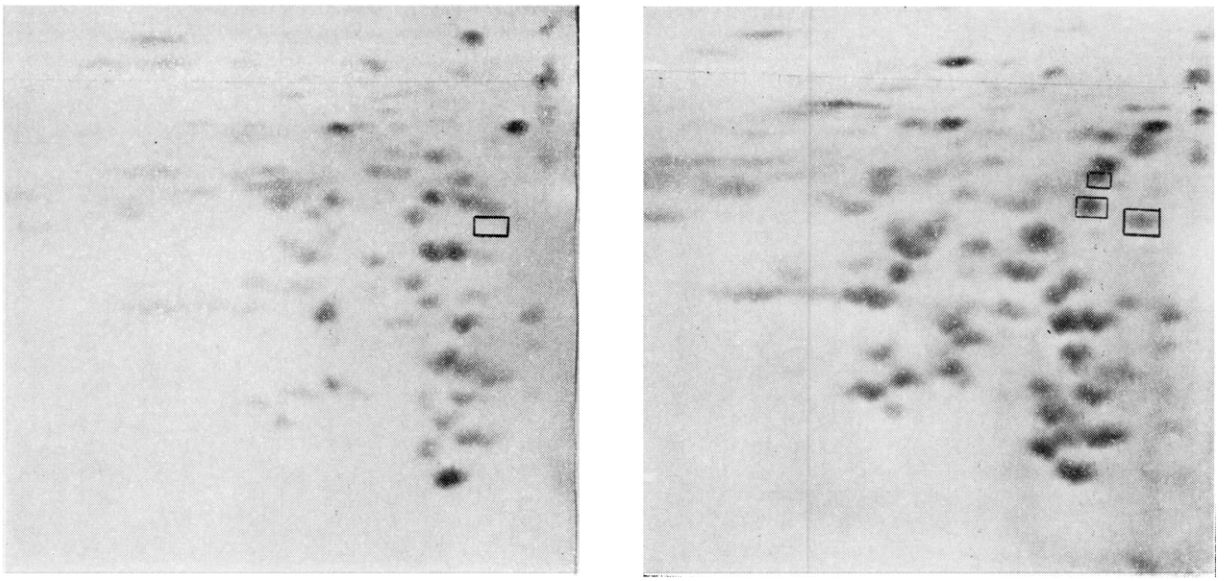


G H

FIGURE 2: Peptide maps comparing trypsin digests of μ chains of reactive proteins (A) macroglobulin 2 and (B) macroglobulin 7 and nonreactive proteins (C) macroglobulin 8 and (D) macroglobulin 14. Below are the respective native proteins (E) macroglobulin 2, (F) macroglobulin 7, (G) maroglobulin 8, and (H) macroglobulin 14. The square marks the single reactive subclass specific peptide (A + E), the double-reactive subclass specific peptide (B + F), the missing nonreactive subclass specific peptide (C + G), and the nonreactive subclass specific peptides seen in three nonreactive proteins (D + H).

rived from each of the two chains in the pepsin digest. The pepsin digests generally contained 35–40 peptides which stained with ninhydrin. Of these 15–18 were clearly derived from the light chain and 15–20 from the μ chain. In each instance, a few peptides (two to four) appeared to be common to the μ and light chain of the protein under study. Approximately one-half of the peptides present in the μ chain were lost following peptic digestion. The missing peptides were primarily those common to all of the μ chains studied and probably came from the carboxy-terminal half of the μ chain which corresponds to the Fc fragment of the γG-globulin.

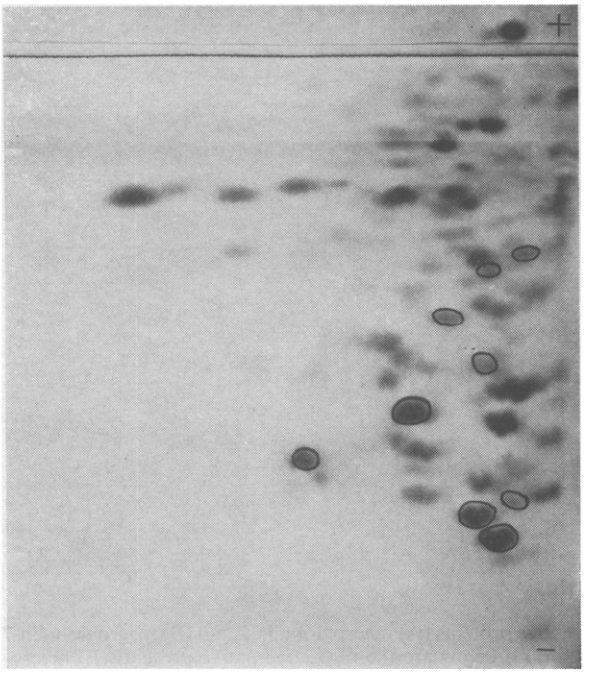
Figure 3 shows the peptide maps of the μ and light chains and peptic digests of two representative proteins. Ten of the μ-chain peptides were common to all of the nine peptic digests studied. (One was missing from one protein only.) The remaining μ-chain peptides (which ranged from five to ten) were seen in one or more of the peptic digests. One of these was present in six of the proteins, but none of the others was seen in more than four of the nine peptic digests studied. Thus, the peptic fragment contained most of the variable peptides, but only about half of the peptides common to all of the μ chains.

Comparisons of μ Chains of Two Serologically Distinguishable μ-Chain Subgroups. One region of the μ-chain maps, indicated by the square in Figure 2, was of particular interest in that the appearance of a small number of peptides permitted the gouping of the proteins into at least three and possibly four chemically distinguishable groups which correlated with the previously described serologically defined subclasses (Franklin and Frangione, 1967). Five of the maps prepared from μ chains of proteins belonging to the minor reactive subgroup had a single peptide marked by the square (Figure 2A), while the other five contained two peptides in close apposition in this region (Figure 2B). The possibility that this was an artifact of the preparation is ruled out by the presence of a similar peptide or pair of peptides in the maps of the native proteins from which the heavy chains were prepared (Figure 2E,F).

In contrast, none of the 15 maps prepared from proteins belonging to the major nonreactive group contained a peptide in the same region. Twelve of these had no ninhydrin-positive material in this region (Figure 2C,G) while three had an appearance similar to that shown in Figure 2D,H. Here again the appearance of the peptides in this region was identical in the maps of the intact proteins, thus indicating that this finding was not a result of the preparative procedures but that it reflected some basic structural features of the protein.

Immunologically pure Fc and Fab fragments were obtained in four instances. Two of the proteins belonged to the reactive and two to the nonreactive groups. None of the fragments prepared from the nonreactive proteins gave precipitin lines with the antiserum to the reactive group, and peptide maps of the Fab fragments prepared from them failed to yield the peptides characteristic of the reactive proteins. Insufficient amounts of the Fc fragment from the nonreactive proteins were available for structural studies. When fragments prepared from two reactive proteins were studied, only the Fab fragment gave a precipitin line with the antiserum to the reactive proteins while the Fc fragment failed to precipitate. In the peptide maps from these two proteins (Figure 4), the characteristic peptide was found only in the Fab fragment and was absent from the Fc fragment.

Further characterization of the subgroup specific



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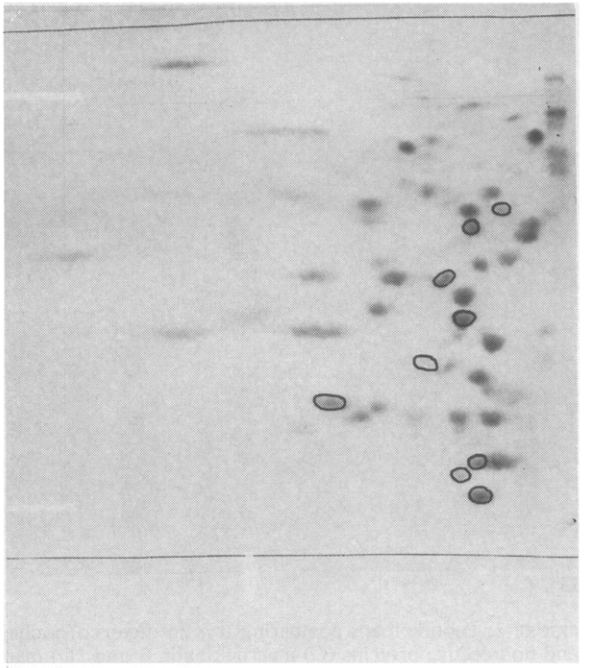

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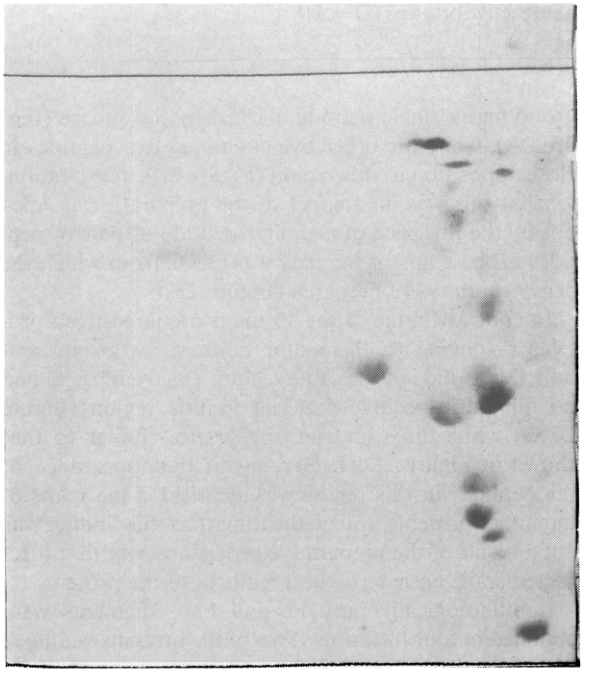

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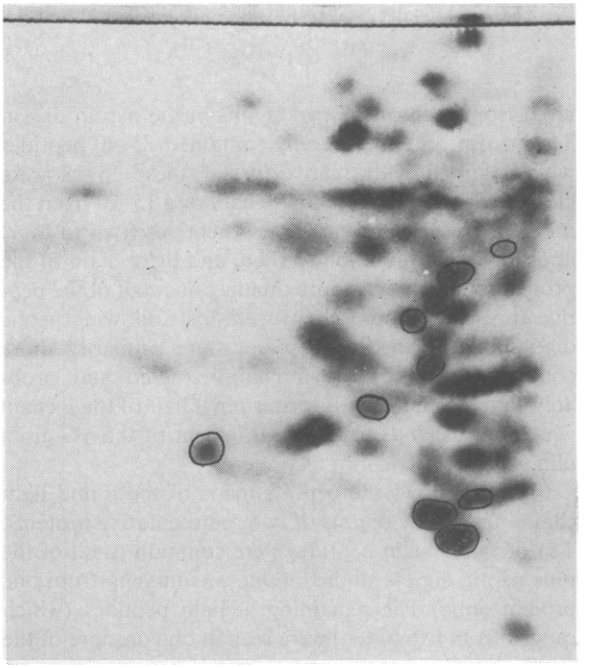
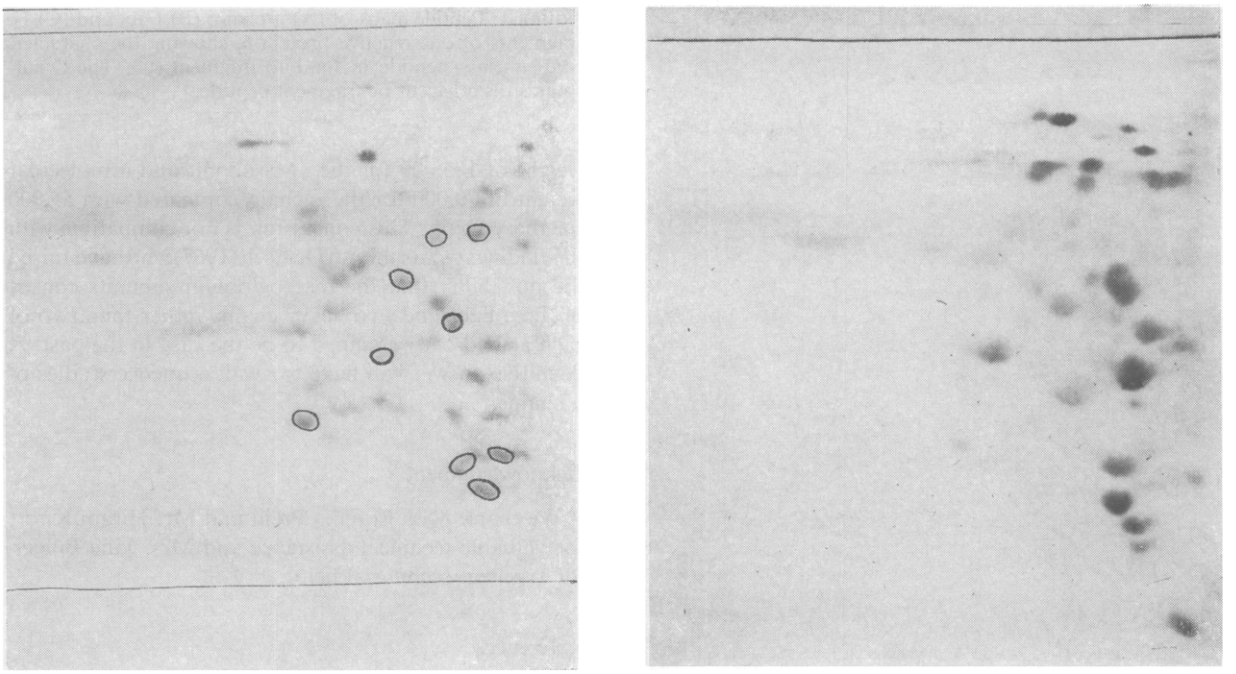


D

peptides was attempted by staining the maps for arginine and histidine. The heavy chains contained 18–22 peptides that stained for arginine. Neither of these two amino acids was found in the peptides characteristic of the two subclasses.

Discussion

The results of this study permit two major conclusions to be drawn. Firstly, the finding of a group of peptides common to all of the μ chains and others present in only a small number of them indicates that the over-all structure of the μ chains is similar to that of the γ chains and the light chains, and that the μ chains, too, appear to contain a common and a variable region. It is not possible without additional studies on the isolated peptides to provide an exact estimate of the size of the common and variable regions. However, based only on the number of peptides rather than their size, it would appear that approximately three-fourths of the heavy chain is common to all μ chains, and that the region of variability can make up at most 25% and probably less of the heavy



E F

FIGURE 3: Peptide maps of trypsin digests from different subunits prepared from two macroglobulins. (A–C) Macroglobulin 7 (A) pepsin digest, (B) μ chain, and (C) light chain. (D–F) Macroglobulin 14: (D) pepsin digest, (E) μ chain, and (F) light chain ○ marks the ten common heavy-chain peptides in the pepsin digest.

chains. While it seems likely that this includes the amino-terminal end of the heavy chain, a direct demonstration of this fact will have to await the detailed sequencing of a number of heavy chains or Fd fragments.

A second point that has been clearly established is related to the two serologically distinguishable subclasses of μ chains (Franklin and Frangione, 1967). The results of this study indicate that these two subclasses differ in their primary structure, and that there are at least two chemically distinguishable variants within each of the serologically defined subgroups. As was the case with the four γ chain subclasses (Frangione *et al.*, 1966; Milstein, 1966a,b; Porter 1967), the serologically detectable differences reflect subtle changes in the primary structure of the heavy chains belonging to the two subclasses. These differences appear to involve only small regions of the μ-polypeptide chain, presumably in the Fd fragment. In the case of the μ chains, reagents to properly define the subclasses have been difficult to prepare, and those currently available may not define and distinguish all the existing subclasses. Consequently, the observations that the reactive as well as the nonreactive subgroups can each be divided further into two chemically distinguishable subgroups suggests that additional subclasses exist. On the other hand, the possibility hat these differences reflect genetic or allotypic variants cannot be excluded, and a definitive answer to this question will have to await additional studies using other chemical and serologic techniques.

There is little doubt that the peptide related to subclass specificity resides in the heavy chain and that it expresses itself in the Fab fragment produced by proteolysis. Consequently, it must be in the part corresponding to the Fd fragment of the heavy chain. While the precise localization in the Fd fragment will have to await the elucidation of the amino acid sequence of proteins belonging to the two classes, it seems likely that it will eventually be located in the constant part of the Fd fragment.

The precise biologic significance of these structural differences is under investigation. Preliminary studies have failed to show any differences in carbohydrate content or complement fixing ability between three proteins belonging to each of the two antigenic subclasses (E. C. Franklin, unpublished observation). Demonstration of the existence of genetic polymorphism analogous to that known to exist in the γ chains will have to await the production of sufficient amounts of reagents to permit accurate quantitation of both subclasses in large numbers of normal sera. Such reagents are not available at this time.

Of interest is the striking resemblance noted between the peptide maps of the native proteins and the heavy chains. The lack of more than a few additional peptides in most cases suggests an even greater homology between the μ chains and light chains than has previously been reported for light chains and γ chains (Hill *et al.*, 1966). However, the restoration of most of the light-chain peptides by the addition of a larger proportion of light chains makes it more likely that light chains make up a smaller fractions of the molecule and that some of the peptides are therefore present in amounts too small to detect. Such a view would be consistent with the data of Miller and Metzger (1965) who estimated a molecular

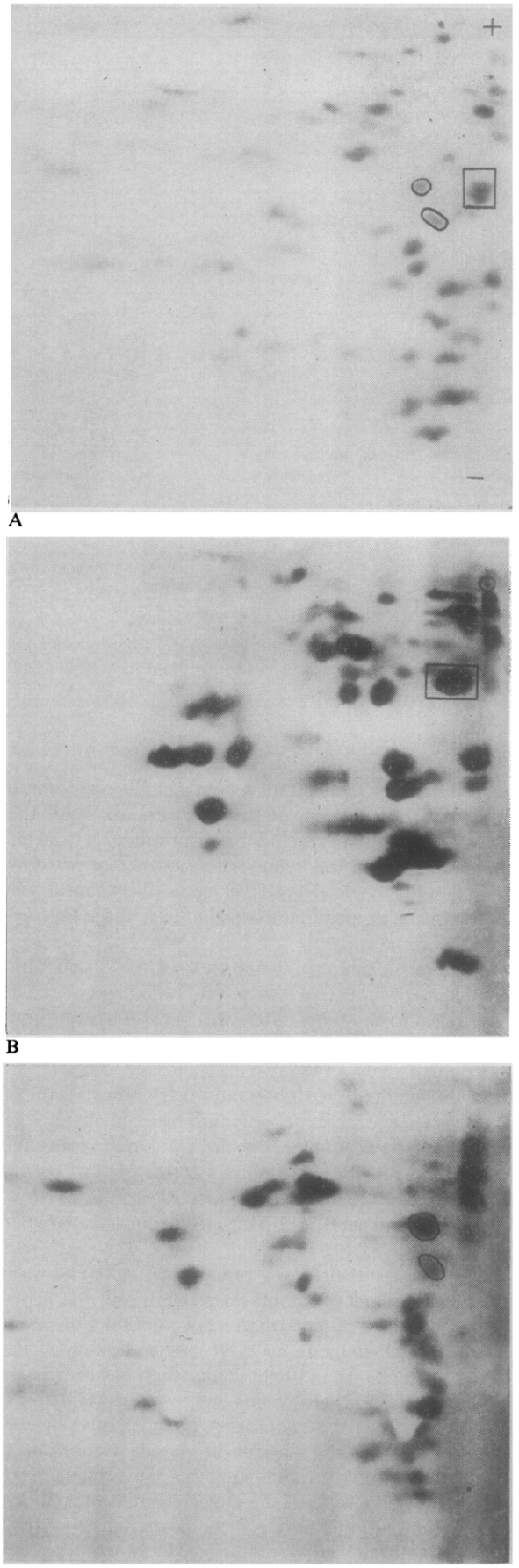


FIGURE 4: Peptide maps of (A) μ chain, (B) Fab, and (C) Fc fragments of one reactive protein 6, showing the characteristic subclass peptide in the Fab fragment (□). The ○ surrounds the adjacent Fc fragment peptide.

weight of 185,000 for the γM subunit and a molecular weight of 70,000 for the μ chain compared with 55,000 for the γ chain. This conclusion is not compatible with the findings of Suzuki and Deutsch (1967) who have raised the possibility that the macroglobulin subunits consist of three light and two heavy chains rather than two of each as had been assumed to be the case in the past. A definitive answer will have to await sequence studies of μ chains.

Acknowledgment

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Protein–Carbohydrate Interaction. XVIII. The Preparation and Properties of Acetylated Concanavalin A, the Hemagglutinin of the Jack Bean*

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ABSTRACT: Concanavalin A, the phytohemagglutinin of the jack bean, was acetylated with sodium acetate and acetic anhydride; 84% of the amino groups and 31% of the phenolic hydroxyl groups were acetylated. The modified protein demonstrated no change in reactivity toward antibodies to the native protein as judged by two-dimensional agar gel diffusion. However, the electrophoretic mobility was changed and the ability of the protein to bind the Mn^{2+} ions (required for activity) was greatly diminished.

The activity of acetylated concanavalin A was investigated in detail. Agar gel and quantitative precipitation studies showed that the reaction of acetylated concanavalin A with the mannan from *Saccharomyces cerevisiae* was almost identical with that displayed by the native protein. However, the interaction with dextran B-1355-S was diminished slightly and that with the levan from *Aerobacter levanicum* was greatly decreased. Acetylated concanavalin A also failed to bind to Sephadex G-50 and a cross-linked levan even when these gels were equilibrated with 0.001 M $MnCl_2$. Inhibition studies with the acetylated protein using several representative inhibitors of the concanavalin A–dextran B-1355-S interaction revealed no change in the specificity of the modified protein. Unlike the native protein, acetylated concanavalin A required high NaCl concentrations (greater than 0.8 M NaCl) before displaying full activity. However, varying the NaCl concentrations from 0.2 to 0.6 M had little effect upon the specific viscosity of the modified protein. It is concluded from these studies that significant acetylation of free amino groups and phenolic hydroxyl groups of concanavalin A yields a modified protein which still retains considerable activity (capacity to precipitate specific polysaccharides) and whose specificity is not altered. This is suggested to mean that free amino groups and many of the tyrosyl residues are not important in maintaining the structural integrity of the protein and its combining sites. It would also appear that these residues are not directly involved in the binding of carbohydrates by concanavalin A.

The phytohemagglutinin of the jack bean, concanavalin A (Sumner and Howell, 1935), has been shown to form a specific precipitate with a variety of biological macromolecules. These include polysaccharides, such as glycogens, dextrans, yeast mannans, amylopectins, and certain levans (Sumner and Howell, 1936; Cifonelli *et al.*, 1956; Manners and Wright, 1962; Goldstein *et al.*, 1965a; Goldstein and So, 1965; So and Goldstein, 1968; L. L. So and I. J. Goldstein, 1968, manuscript in preparation), various serum glycoproteins (Nakamura *et al.*, 1960, 1965; Harris and Robson, 1963; Leon, 1967; I. J. Goldstein, L. L. So, Y. Yang, and Q. Callies, 1968, manuscript in preparation), and a number of carbohydrate–bovine serum albumin conjugates (Goldstein and Iyer, 1966).

The stereochemical requirements of the combining sites of concanavalin A have been investigated in detail by examining the extent to which a wide variety of low molecular weight carbohydrates inhibited the concanavalin A–polysaccharide interaction (Goldstein *et al.*,

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