HYBRID FORMATION BETWEEN HEAVY IgG CHAINS AND A BENCE - JONES PROTEIN

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ABSTRACT

HYBRID FORMATION BETWEEN HEAVY IgG CHAINS

AND A BENCE-JONES PROTEIN

By

Mary Makri

Hybridization of heavy immunoglobulin chains from human IgG's mixture with homogeneous Bence-Jones proteins was studied.

In one case the free SH groups of the heavy chains were alkylated. Then the hybrids formed were non-covalently bound. In the other case the SH groups were left free to make covalent bonds in the newly made hybrids.

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Mary Makri

A THESIS

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Dedicated to my mother, Helen.

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LIST OF ABBREVIATIONS

H = Heavy Chains

L = Light Chains

aa = Amino Acid

B. J. = Bence-Jones

IgG = Immunoglobulin G

Fab (t) = Fab obtained after trypsin digestion

M. W. = Molecular Weight

REVIEW OF LITERATURE AND INTRODUCTION

Antibodies have been known since the late of 19th century. In 1917 Karl Lands Landsteiner demonstrated that animals could form antibodies against certain small organic chemicals of known structure. In 1959 Gerald M. Edelman at Rockefeller Institute found that antibody molecules consist of polypeptide chains or protein subunits of more than one kind, and that the chains could be separated from one another by chemical means. These chains were called light (L) chains and heavy (H) chains because of the difference in their size.

Different classes of immunoglobins can be separated from serum by physical and chemical techniques and five classes of immunoglobins have been defined according to their general properties and the classe of their heavy chains (γG, γA, γM, γD, γE). In contrast to the heavy chain classes, the two major classes of light chains k and λ are found in all immunoglobins. Although they share the same overall structure, immunoglobins within a single class and subclass are a heterogeneous mixture of chemically different molecules (e.g. unequivocal aminoacid sequence).

Studies have been done in the determination of the aa sequence (Lennox and Cohn 1967, Cohen and Milstein 1967, Edelman and Gall 1969) and on higher levels of organization

in immunoglobin molecules; their size and shape and intimate aspects of their internal folding.

Molecular weight determinations were made from ultracentrifugal, osmotic pressure, light scattering and low-angle X-ray scattering studies. For γG-globulins which have been studied in Human, rabbit, and horse the M.W. is given as 143-150 x 10^3. In the M.W. determination there is a concentration depencence problem which arises from the effects of aggregates in the γG preparations resulting in lower values. The problems of aggregation and limited solubility of the heavy chains of γG-globulins (and other immunoglobin classes) in neutral aqueous solvents have meant that reliable values of their M.W. size have only been obtained in dissociating solvents (this has also been true for the L chain).

Values of heavy to light chain ratios, weights of single chains and z-average M.W. were compared with similar values for calculated equimolar mixtures of chains of assumed size. The results were compatible only with a four-chain model. The 2H chains with a M.W. of 50-65 x 10^3 each and the 2L chains between 20-25 x 10^3 M.W. each. On the other hand, with the determination of the complete amino acid sequence of the H chains (Edelman and Gall 1969) of one γG molecule a M.W. of 48,600 can be calculated (not including carbohydrates which could bring it up to 50,100).

The overall shape of γG-globulins has first been considered by Edelman and Gally (1964) to be a rigid rodlike

structure with the antibody-combining sites at the extreme ends of the rod (Fig. 1).

The two identical L chains and two identical H chains which constitute the γG-immunoglobin molecule interact via noncovalent forces and a single disulfide bond. Two light chain-heavy chain pairs of half-molecules are linked together by noncovalent interactions and by two neighboring disulfide bonds between the heavy chains. (The organization of the whole molecule is shown in Fig. 2).

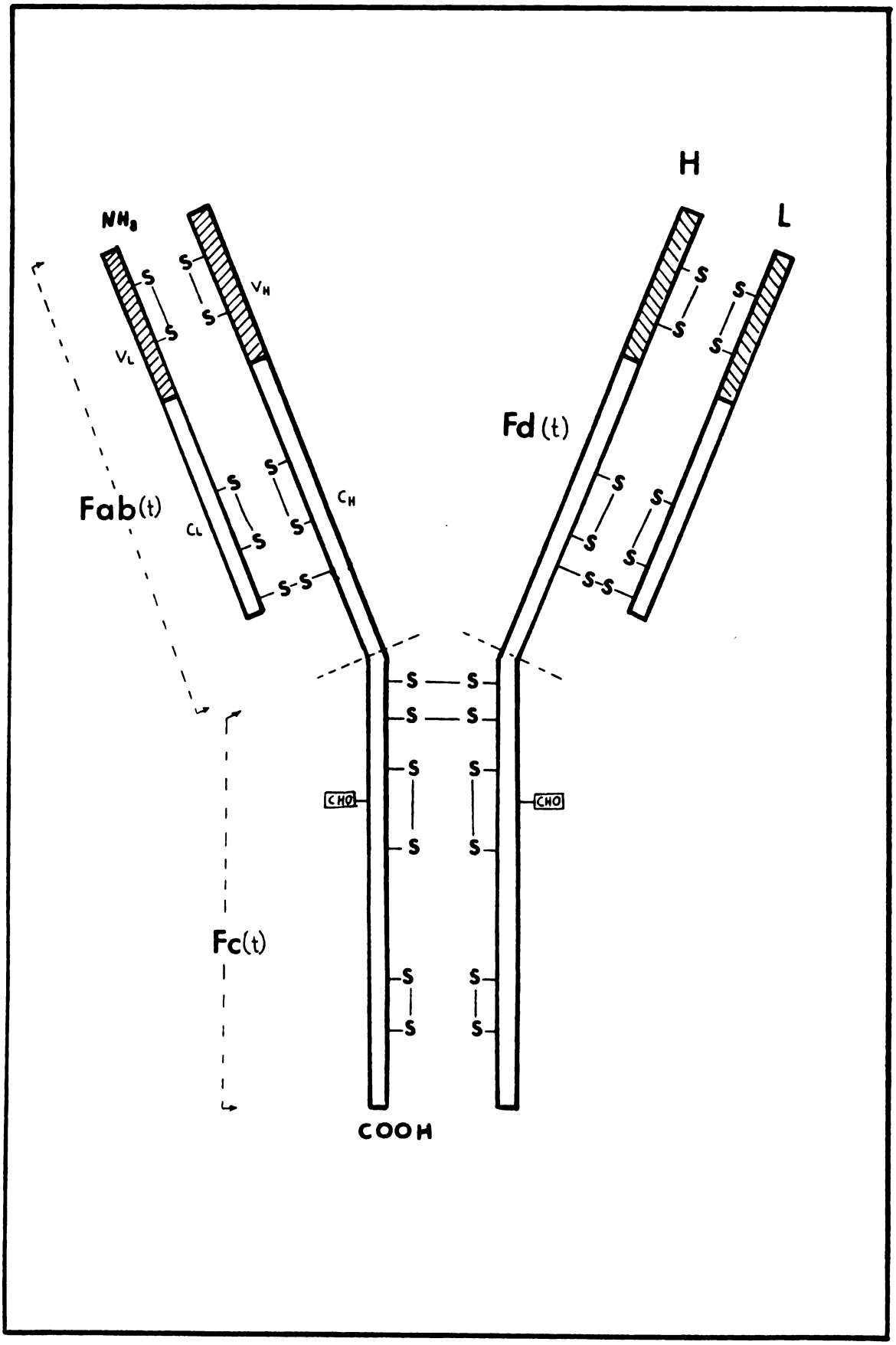
With trypsin or papain digestion the molecule gives two kinds of fragments two Fab's or antigen-combining sites that consist of the whole light chains plus the Fd or variable amino acid portion of the heavy chain plus a portion of its constant region, and two Fc's or the remaining constant amino acid region of the heavy chains. On both L and H chains the Fab (t) fragments (i.e. NH_2 terminal) have a variable region which is homologous and similar in length for both L and H chains. The rest of the chains consist of the constant region (definited aa sequence for each kind of immunoglobin). Each constant region and each variable one contains an interchain disulfide bond. The half-cystines participating in this bond have a distance of 10 aminoacid residues between them.

On the basis of antigenic analysis and aa sequence studies, light chains can be divided into two classes: K and λ. Their carboxyterminal halves are identical (constant regions). The aminoterminal halves are variable and contain

Figure 1.--The rigid-rod model of Edelman and Gally for the IgG molecules.

Figure 2.--Overall arrangement of chains and disulfide bonds of the γG immunoglobin.

CHO: carbohydrates
Fab(t) and Fc(t): refer to fragments produced by trypsin which cleaves the heavy chain as indicated by dashed lines above half-cystinyl residues.
V_H, V_L: variable regions of heavy and light chains.
C_H, C_L: constant regions of heavy and light chains.


H
L
NH2
VH
VL
Fd (t)
Fab(t)
CH
CL
CHO
CHO
Fc(t)
COOH

an area of extreme sequence variability termed "hypervariable region." (Three of them have been determined between the residues [24-34], [50-56] and [89-97]). These hypervariable areas may direct binding with the antigen as Wn and Kabat suggested (1970). The areas of basic tertiary structure are essential for the expression of any antibody specificity while the hypervariable regions would provide the extensive variations required for the expression of the many different specificities of antibody molecules. The heavy chain variable region also involves one hypervariable region [31-37] and both these of the hypervariable regions of the L and H chains are located at the same position. This could be evidence for a common evolutionary origin for both regions.

The size of the combining region has been calculated to be large enough to accomodate 4 aa residues as Sela has shown by using cross precipitation with peptides of known structure attached to large proteins (e.g. RNase)(1969).

In the absence of detailed X-ray analysis, much of the information on the configuration of immunoglobins in solution has come from ORD and CD studies. Much of the ORD data on immunoglobins has been performed on γG-globulins. The spectra of different species are almost similar (Dorrington and Tanford 1968). These spectra are characterized by:

a. Low level of rotation throughout the wavelength range.

b. Single maximum absorption between 204-210 mμ with a crossover (zero rotation)near 220 mμ.

c. Cotton effect minima near 230, 225 and 198 mμ.

d. In γG only, a Cotton effect of low rotatory strength at 240 mμ.

Based on the ORD and CD data, it seems fairly certain that immunoglobins don't have significant amounts of α-helical regions.

Isolated heavy chains can bind homologous antigens but the equilibrium constant is two times smaller than that of the whole molecule. The L chains also have measurable binding activity. The two possible explanations for the observation of specific affinity with reduced a binding constant are:

1. The binding site of the native antibody involves both the chains (H and L).

2. The binding site involves only one chain and the other chain is required to maintain the molecule in a reactive conformation.

The structural studies of the polypeptide chains of the immunoglobin molecules led to a clue that made it possible to bypass the problem raised by the instrinsic heterogeneity of antibodies. The clue concerned the nature of certain homogeneous proteins made by tumors of plasma cells (which produce the most antibodies).

Knowledge of the tumors occured in 1847 when Henry Bence Jones (a physician in London) published a paper titled: "On the New Substance Occuring in the Urine of a Patient with Mollities Ossin." Although 700 papers on the subject appeared, Bence-Jones proteins remained a kind if chemical

and biochemical curiosity except for their diagnostic help in the malignous disease of plasma cells called "multiple myeloma." In 1959 it was found that B. J. proteins were homogeneous light chains made by the tumor but not incorporated into whole molecules.

No two individuals produce the same B. J. proteins but have a singular structure. Each molecule has about 214 aa and the polypeptide chain contains one constant and one variable region (after the 109th aa).

The M.W. of these proteins, which excreted in grams per day from the urine of the patients, is about 23×10^3.

Many studies have been done on the conditions of reconstitution of 7S γ-globulin molecules (associated through non-covalent interactions) from separated polypeptide chains (M. Fougereau and G. M. Edelman, 1963). The molar ratio of H and L chains used in the reconstituted molecules approached those of native γ-globulin (1:1) or an excess of L chains was mixed with H chains.

The H and L chains had been isolated from IgG molecules after reduction (mercaptoethanol) in the absence of urea (G. M. Edelmann and M. D. Poulik, 1961) for two hours followed by addition of iodoacetamide (alkylation). The separation of the H and L chains was done by gel filtration after the addition of propionic acid. Reduced and alkylated L and H chains having different isotopic labels were mixed while still in propionic acid and then dialyzed against pH 8.0 buffer.

Similar studies have been done by I. Björk and C. Tanford (1971) on isolated reduced and carboxymethylated H and L chains involving separation in propionic acid and followed by reconstitution after renaturation in pH 5.5 buffer.

On the other hand the preferential recombination of autologous polypeptide chains were studied by Mannik (1967) and Grey (1965) who showed that there exists a specificity in these interactions between H and L chains such that the heavy and light chains of human G-myeloma protein tend to reassociate even if other free L chains are present. This preferential recombination of autologous H and L chains occurs even at an 80 fold molar excess of heterologous myeloma L chains.

To show existence of similar preference at the subclass level, studies by J. Gergely-G. A. Mergyesi and Maria Pakh-Csecsi Nagy and Eva Puskas (1973) have been done. This work on heterologous H and L chains originating from IgG myeloma proteins of identical and subclasses proved that chains which have been previously folded together into molecules belonging to the same subclass, associate more easily with each other than H and L chains prepared from myeloma proteins of different subclasses. So a preference in association of H and L chains exists at the subclass level.

One of the most exciting problems in molecular Biology is the relationship between antigenic specificity of

antibodies and their organization at the several levels of protein structure and especially on the aa sequence level.

Various theories of antibody formation ascribe different roles to the immunogen during immunization. They are divided into two categories: Selective Theories and Instructive Theories (Davis, B.D., Dulbecco R, Eisen, H.N., Leinsberg W., 1967).

According to the first theory (Clonal Selection Theory: Burnet and Kind), antibody molecules are already made at a low level (in different cells) and when a specific antigen is present a clone of the appropriate kind of special cells develops, giving a greater amount of the antibody molecules needed.

Instructive theories in contrast assume that the immunogen helps to shape the corresponding antibodies which could not be made in its absence (except as a rare and fortuitous event). It means that by rapid mutations of the corresponding genes or by recombinations of genes responsible for the variable regions, formation of specific antibodies occurs after the antigen binds.

This work is part of experiments designed to test another hypothesis which might explain the antibody diversity as a result of transpeptidation reactions. According to this theory, the antibody structure (primary) is determined by the antigen structure that is responsible for the transpeptidations and gives to the antibodies its specific structure. In summary this theory includes:

1. Existence of "proantibody" molecules which can conbine weakly with antigens and produce some disorder in some regions in the structure of proantibodies.

2. The disordered regions are easily proteolized by proteolytic enzymes resulting in the formation of gaps that will be repaired (analogous to DNA repair) by transpeptidations resulting in more ordered structures (stable molecules).

These molecules are specific antibodies. These modified chains will be used as "template" with transpeptidation processes for new such antibodies formation when the antigen is no longer present.

In order to test this hypothesis it will be necessary to purify L chains (Bence-Jones proteins) first, followed by preparation of H chain mixtures of human gamma globulin derived from pooled blood.

The next step would be to associate them non-covalently and covalently in order to form hybrids which will be prepared from IgG H chains and B. J. proteins as L chains.

These hybrids will then be used for testing this hypothesis assuming that there is similarity with the "proantibodies" molecules.

MATERIALS AND METHODS

A Bence-Jones protein was obtained from the urine of a patient with multiple myeloma. IgG (human immunoglobins, Cohn fraction II) were obtained from Sigma. Acrylamide (for electrophoresis), methylenebisacrylamide, N, N, N' N' tetramethylethylenediamide (T E M E D) and ammonium persulfate were Canalco products. Analytical grade $NaH_2PO_4 \cdot H_2O$ $NaH_2PO_4 \cdot 7H_2O$ and Propionic acid were obtained from Mallinckrodt Chemical Works, St. Louis, Glacial acetic acid and methanol were reagent grade chemicals. Thioglycerol had been distilled, and double distilled water was used in all experiments.

Light (L) Chains (Bence-Jones Protein) Preparation

A homogeneous B. J. protein was obtained from one day's urine of a patient with multiple myeloma by repeated precipitation with 60% saturated ammonium sulfate. The precipitate was dialyzed against doubly distilled water and lyophillyzed.

Preparative chromatography

The procedure used is basically the same as given by Bernier and Putnam (1964). It includes gel filtration

(Sephadex G-100) and ion-exchange chromatography (DEAE-Sephadex A-50). In addition a second gel filtration step was done (Sephadex A-25).

a. Ion exchange chromatography.

Column: DEAE-Sephadex A-50, 50 cm x 1.8 cm. Some Sephadex G-25 was added on the top in order to give a stable surface.

Buffer: Potassium Phosphate 0.02M pH 8.0 (i.e. 0.02M K_2HPO_4 and 0.02M KH_2PO_4 adjusted to pH 8.0).

Flow Rate: 36 ml per hour.

Sample: 1.0 g lyophillized protein in 8 ml buffer. The column was washed with 1 liter buffer before the sample was layered.

b. Gel Filtration (first).

Column: Sephadex G-100, 148 cm x 0.9 cm. Sephadex G-25 was added on the top as in DEAE step.

Eluate: NaCl 0.15 N.

Flow Rate: 5.5 ml per hour.

Sample: .8 g in 10 ml of 0.15 N NaCl.

c. Gel Filtration (second).

Column: Sephadex G-25, 98 cm x 2 cm.

Eluate: Boiled water.

Flow Rate: 60 ml per hour.

Sample: Pooled protein fractions from first gel filtration.

Analytical Technique

Acrylamide Gel Electrophoresis (7.5% acrylamide gel final concentration). Acrylamide gel electrophoresis was carried in vertical apparatus.

Solutions for preparation of gels:

Solution A: 0.8M Sodium Phosphate buffer pH 7.1, 0.23 ml TEMED per 100 ml of solution.

Solution B: 20% acrylamide, 0.735% methylenebisacrylamide.

Solution C: 28% ammonium persulfate freshly made.

Recipe: Mix 1 part A, 3 parts B, 1 part 20% glycerol, 2 parts C and 1 part H_2O.

Electrophoresis buffer: Upper and lower reservoirs of Electrophoresis Unit were filled with 0.1M sodium phosphate buffer pH 7.1.

Staining Solution: 0.4% Coomasie brilliant blue, 10% trichloroacetic acid and 33% methanol.

Destaining Solution: 10% trichloroacetic acid and 33% methanol.

The electrophoresis was performed at a constant current of 8 m A per gel. Bromophenol blue (0.05%, 3µl) was used as a tracking dye. Sample: 5 µg protein in 5 ml.

Heavy Chain (H) Separation

Reduction and Alkylation of the IgG

I. Björk and C. Tanford's (1971) procedure was basically used. IgG mild reduction was performed in 0.5M tris-buffer pH 8.24 with 0.2M thioglycerol (instead of mercaptoethanol) for two hours at room temperature (the sample had been deaerated and kept in N_2 atmosphere) and was followed by carboxymethylation with an equimolar (0.2M) amount of iodoacetamide for ten minutes. The solution was then dialyzed against H_2O.

Separation of H and L Chains by Gel Filtration in Propionic Acid

A solution of 161 mg of reduced and carboxymethylated IgG in 8 ml of water was made up to 1M propionic acid by the addition of concentrated propionic acid. After two hours the reaction mixture was applied on a Sephadex G-100 column (2.5 cm x 90 cm) in order to separate heavy monomer chains.

Eluting solution: 1 M Propionic Acid.

Flow Rate: 15 ml per hour.

Non-Covalently Bound Hybrids Formation

This includes:

1. Renaturation. The pooled heavy chain fractions from the G-100 filtration were dialyzed against five changes (2,000 ml each) of 0.01 sodium acetate pH 5.5, followed by

three changes (2,000 ml each) of 0.02 M sodium acetate pH 5.5 in 0.1 N NaCl.

2. Removal of aggregates. In order to remove aggregated heavy chains, the solution was concentrated by ultrafiltration through a Diaflo UM 20 E membrane (Amicon) and the concentrate filtrated through Sephadex G-200 (2.5 cm x 88.5 cm column dimensions).

Buffer: 0.02 M sodium acetate pH 5.5 in 0.1 N NaCl.

Flow Rate: 3.7 ml per 40 minutes.

Sample: 11.7 ml.

3. Hybridization. Equimolar amount of B. J. protein and heavy chains were mixed together (approximately the M. W. of B. J. is = 1/2 M. W. of heavy chains) in: 0.02 M sodium acetate pH 5.5 in 0.1 N NaCl. After 42 hours in the cold the sample, 10 ml total volume, 10.3 mg total protein (3.4 mg B. J. proteins + 6.9 mg H chains) was filtered through Sephadex G-200.

4. Gel Filtration of Hybrids.

Column: Sephadex G-200 2.5 cm x 88 cm.

Buffer: 0.02 M sodium acetate pH 5.5 in 0.1 N NaCl.

Sample: 10 ml (0.35 mg per ml protein).

Flow Rate: 3.3 ml per 40 minutes.

5. Analytical Technique. SDS-polyacrylamide gel electrophoresis of hybrids. For this the procedure of Weber and Osborn (1969) was basically used (i.e. 10% acrylamide). The samples before they were applied on the gel were denaturated in 0.1% SDS plus 0.1% trioglycerol at 37°C for two hours.

Covalently Bound Hybrids Formation

1. Reduction of IgG but no alkylation. In 8.2 ml of tris-buffer (0.05 M pH 8.5) 170 mg IgG were dissolved by gentle stirring. The solution was deaerated for a few minutes; after this 0.176 g of redistilled thioglycerol was added (final concentration 0.2 M). The sample was placed in a bell filled with N_2 for 2 1/2 hours at room temperature. The solution was brought up to 1 M in propionic acid by adding 0.592 ml concentrated propionic acid.

2. Separation of H chains by gel filtration.

Column: Sephadex G-100 2 cm x 95 cm.

Eluating solution: 1 m propionic acid and 0.05 M thioglycerol.

Sample: = 9 ml, 170 mg protein.

Flow rate: 5 ml per hour.

To the pooled fraction of H chains (volume 62.5 ml, concentration .78 mg/ml) was added an excess of the B. J. protein. The solution was then dialyzed agains three changes (2 liters each) of 0.1 M sodium acetate, pH 5.5, for 36 hours at 4°C. The dialyzed solution was then concentrated and dialyzed against 0.02 M sodium acetate pH 5.5 containing sodium chloride (0.1 M). Finally it was filtered through a Sephadex G-200 2.5 cm x 90 cm column.

Flow rate: 5.2 ml per 40 minutes.

Buffer: sodium acetate 0.02 M pH 5.5 containing sodium chloride (0.1 M).

Sample: 114 mg protein (48.75 mg H plus 66 mg L chains) in 13 ml buffer.

All the protein determinations in the experiments were based on the absorbance of the proteins at 280 m . Extensions of the columns were used (1 x their length) in order to pack them and all gels and buffers were heated before pouring the columns.

Filtrations and hybridizations performed at 4°C.

RESULTS

Bence-Jones Protein Purification

By gel chromatography it was possible to isolate the major component of the B. J. proteins, which migrated largely as a single band in acrylamide gel electrophoresis. The minor components were not isolated in any instance.

By ion exchange chromatography the monomer was isolated as major component and when the gel filtrations were performed the major component was freed of small amounts of other proteins (Fig. 3 and 4). Acrylamide gel electrophoresis was used for the examination of the homogeneity of the B. J. protein. Figure 5 shows the results of the gel electrophoresis.

Heavy Chain Separation

In order to make non-covalently bound hybrids, heavy chains from IgG molecule must be separated from the light chains and then utilized for the hybrid formation. For this separation it was necessary to break the disulfide interchain bonds (Fig. 2) of the IgG molecule. In the case where the hybrids would not be covalently bound the resulting SH groups, after the cleavage of the disulfide bonds, had to be protected against reoxidation. The reduction and alkylation of the heavy chains can be easily

Figure 3.--The first gel chromatography of B. J. protein monomers (after ion exchange chromatography) on Sephadex G-100 in 0.15 N NaCl. 1.0 g of protein (80 mg/ml) on a 148 cm x 0.9 cm column.

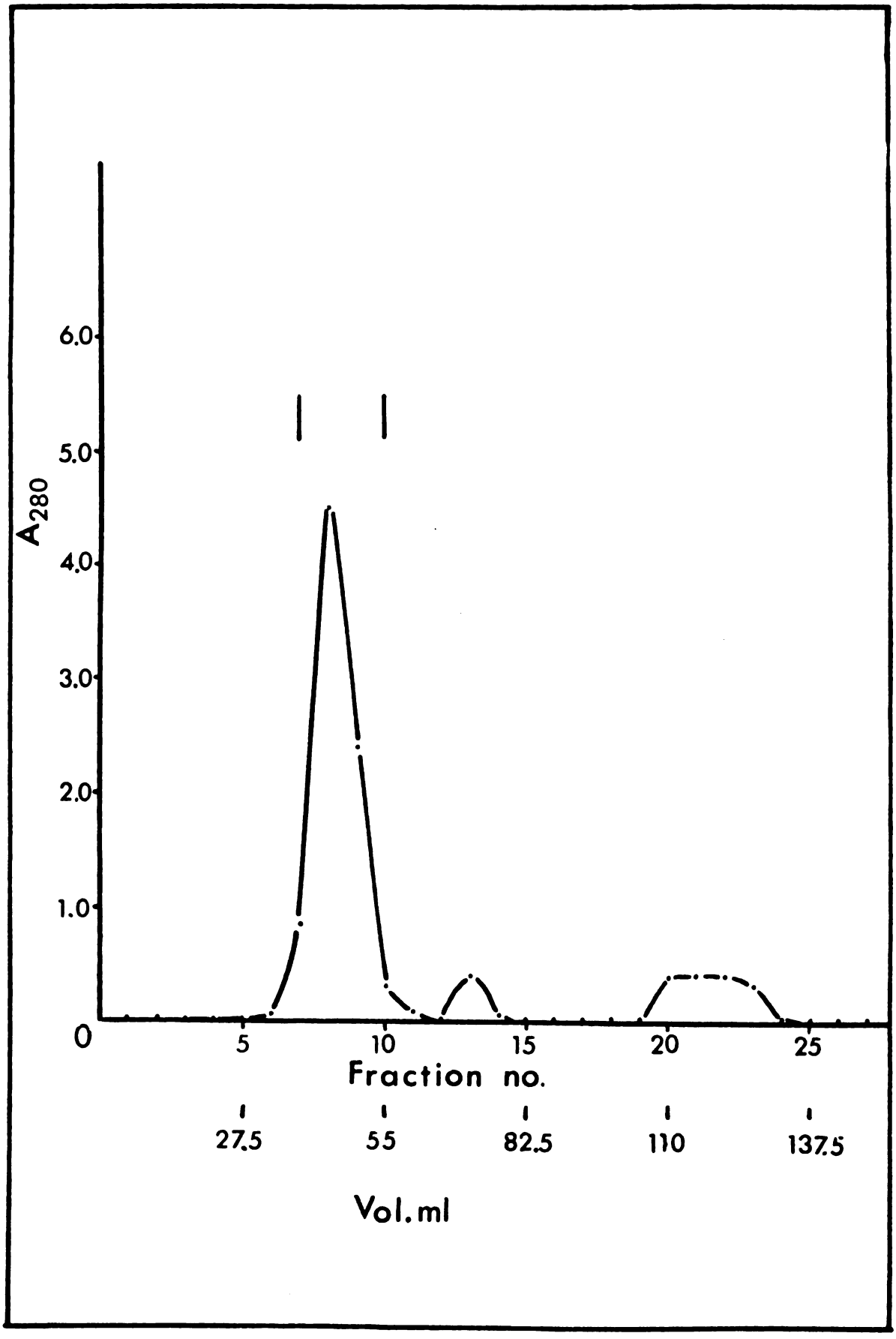

6.0
5.0
4.0
3.0
2.0
1.0
0
A_{280}
5
10
15
20
25
Fraction no.
27.5
55
82.5
110
137.5
Vol. ml

Figure 4.--The second gel chromatography of B. J. protein monomers after the first gel filtration (G-100) on a Sephadex G-25 in H_2O .75 g of protein in 10 ml 0.15 N NaCl on 98 cm x 2 cm column.

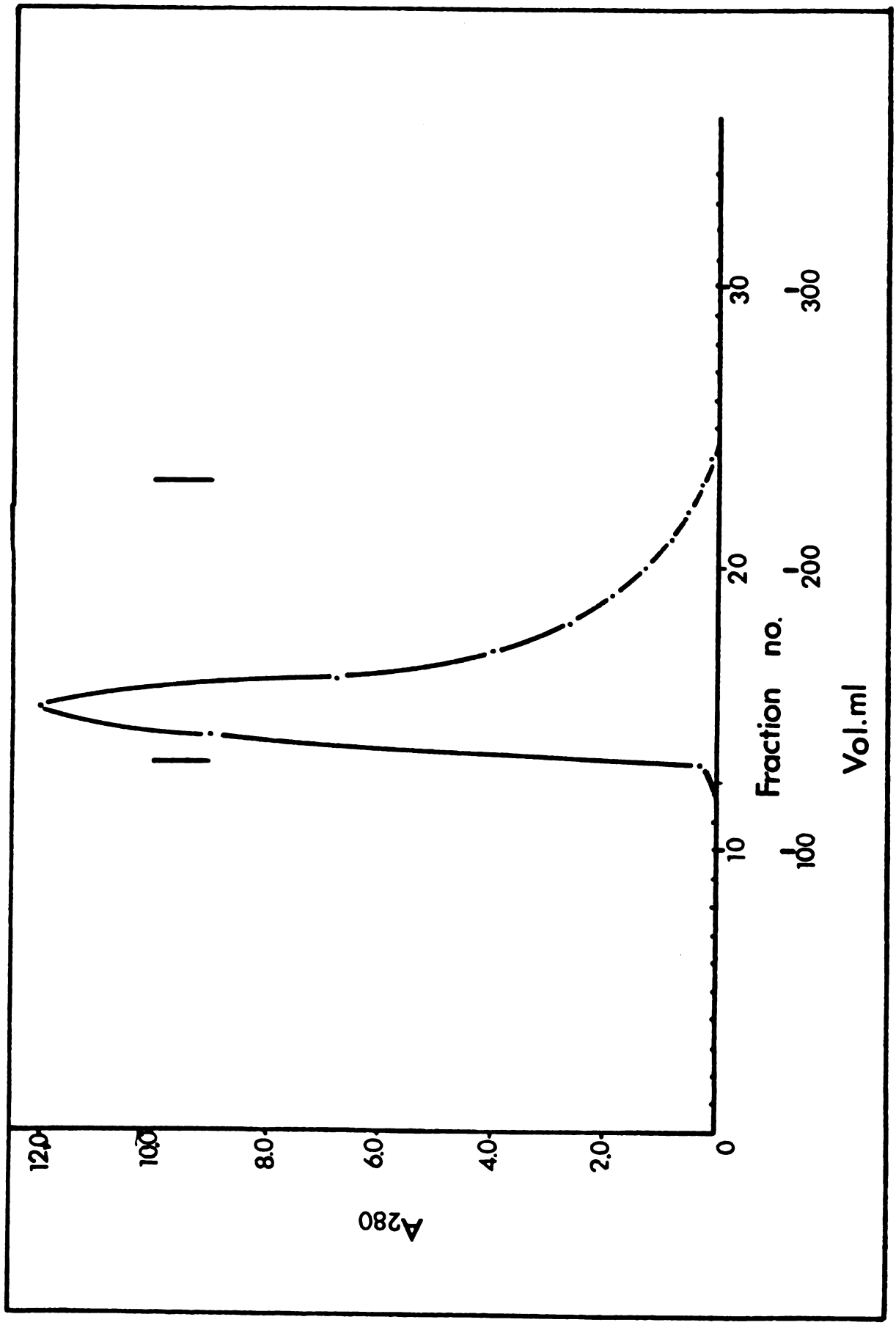
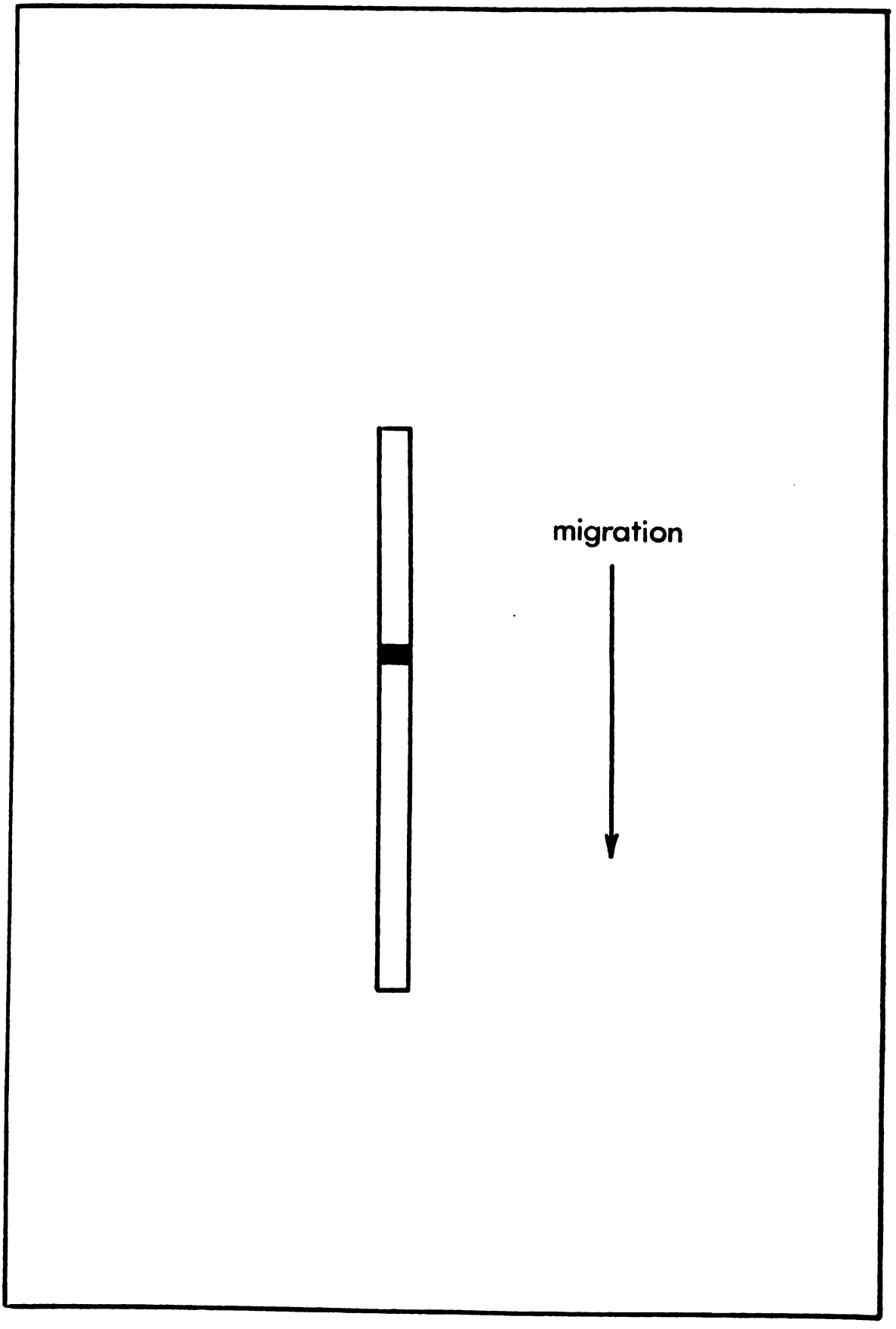

A280
12.0
10.0
8.0
6.0
4.0
2.0
0
10
20
30
100
200
300
Fraction no.
Vol.ml

Figure 5.--Acrylamide gel electrophoresis of B. J. proteins excreted from the daily urine of a patient on multiple myeloma. The protein previously had chromatographed with ion-exchange chromotography and two gel-filtrations.


migration

done under relatively mild conditions (0.2 M thioglycerol and 0.2 M iodoacetaruide). But for the strong non-covalent bonds between the heavy and light chains required quite drastic conditions, eg. 1 M propionic acid. The chains are then separated by gel filtration (G-100) in the dissociation media.

Because the H chains show a tendency to polymerize in IM propionic acid, it was necessary to minimize the time in solution.

Fig. 6 is the gel chromatography for the separation of heavy and light chains. The heavy chain peaks (actually there are three poorly resolved peaks) include polymers of the heavy chain and also aggregates. (Olins D. E. and Edelman G. M. 1964). In order to separate polymers, aggregates, and monomers, one more filtration (G-200) (Fig. 7) has been performed on the pooled fractions (28-47) after they had been extensively dialyzed against 0.1 M sodium acetate buffer pH 5.5 and 0.02 M sodium acetate buffer pH 5.5 in 0.1 NaCl (renaturation) followed by concentration with ultrafiltration through a Diaflo UM 20 E membrane. Fig. 7 shows the separation of the monomer from the polymers and aggregates of heavy chains. The vertical lines indicate those fractions used for further experimentation. The pooled monomerfractions were concentrated and with B. J. protein (which had been kept freeze dried) were mixed together and allowed to hybridize (see methods).

Figure 6.--Gel filtration (G-100) of mildly reduced and carboxymethylated IgG molecule, in 1 M propionic acid. A 10 ml sample (161 mg protein) applied on a 2.5 cm x 90 cm column.

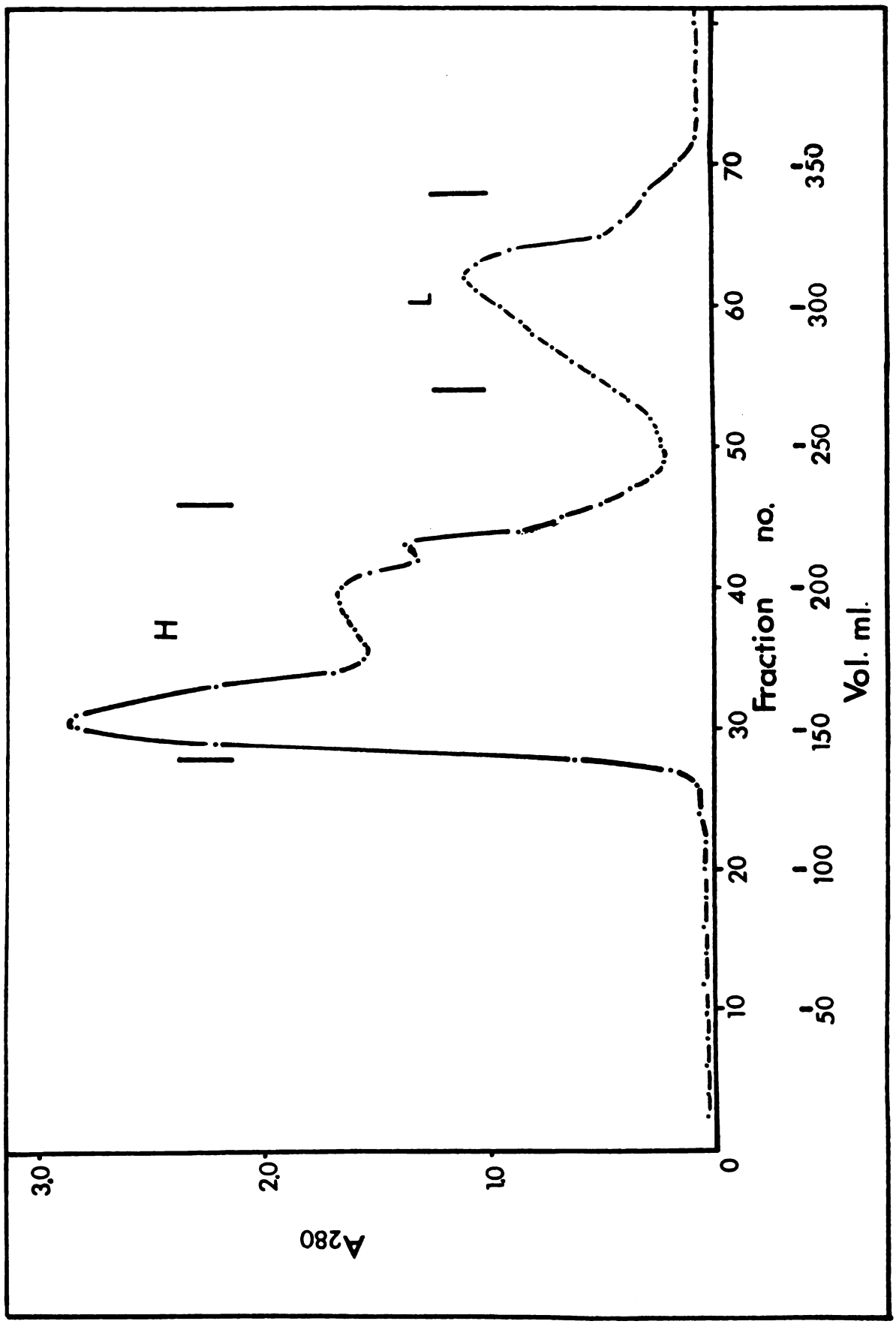

A280
3.0
2.0
1.0
0
H
L
Fraction no.
Vol. ml.
50
100
150
200
250
300
350
10
20
30
40
50
60
70

Figure 7.--Gel chromatograph of reduced and alkylated heavy chains from IgG in sodium acetate 0.02 N pH 5.5 in 0.1 NaCl on a 2.5 cm x 88 cm Sephodex G-200 column.

Flow rate = 3.7 ml per 40 min.

.138 mg. protein per ml sample concentration.

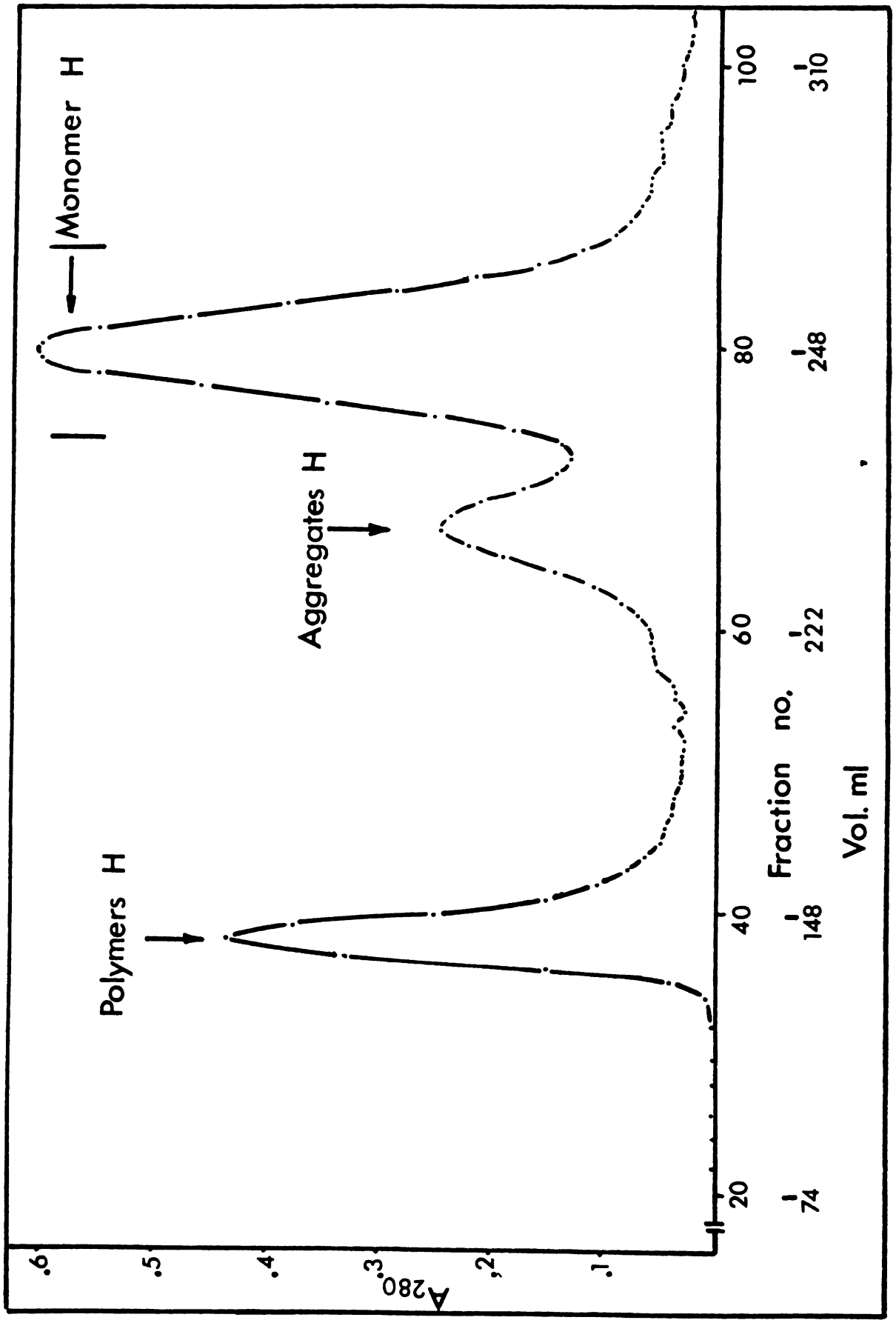

Monomer H
Aggregates H
Polymers H
A280
.6
.5
.4
.3
.2
.1
20
40
60
80
100
74
148
222
248
310
Fraction no.
Vol. ml

Fig. 8 shows the chromatograph G-200 obtained with an equimolar mixture of heavy (from Ig G) and light (B. J. protein) chains. The mixing of the chains had been performed at pH 5.5 after the renaturation of the reduced and carboxymethylated heavy chains. The three peaks obtained A, B, and C are heavy chains which have not been hybridized due to aggregates, not completely removed with the gel chromatography, hybrids, and unhybridized light chains respectively.

A SDS-polyacrylamide gel electrophoresis was performed as a control of the hybridization (peak B). With the thioglycerol and the SDS (sodium dodecyl sulphate) the chains are separated from each other according to their M. W. only. In Fig. 9 the three patterns are the SDS acrylamide gel electrophoresis of peak A, peak B and light chain (B. J. protein) used as standard (their homogeneity had been proved before with the acylamide gel electrophoresis performed) (Fig. 5). The explanation which can be given for the bands is: First gel (peak A). The many bands located above the standard band, which is light chain (B. J. protein on the third gel) showed different aggregates and polymers of the heavy chains which migrate differently because of their different M. W. Because of this aggregation they have not made hybrids with light chains (B. J. protein) which remain free giving the third peak (Fig. 7). The second gel (peak B) gives two bands clearly separated from each other. The one of them (a)

Figure 8.--Gel chromatography on Sephadex G-200 of recombined heavy chains from IgG and B. J. protein.

Column dimensions = 2.5 cm x 88 cm. Buffer sodium acetate pH 5.5 in 0.1 N NaCl.

10 ml sample (0.35 mg per ml protein) applied after 42 hours mixing.

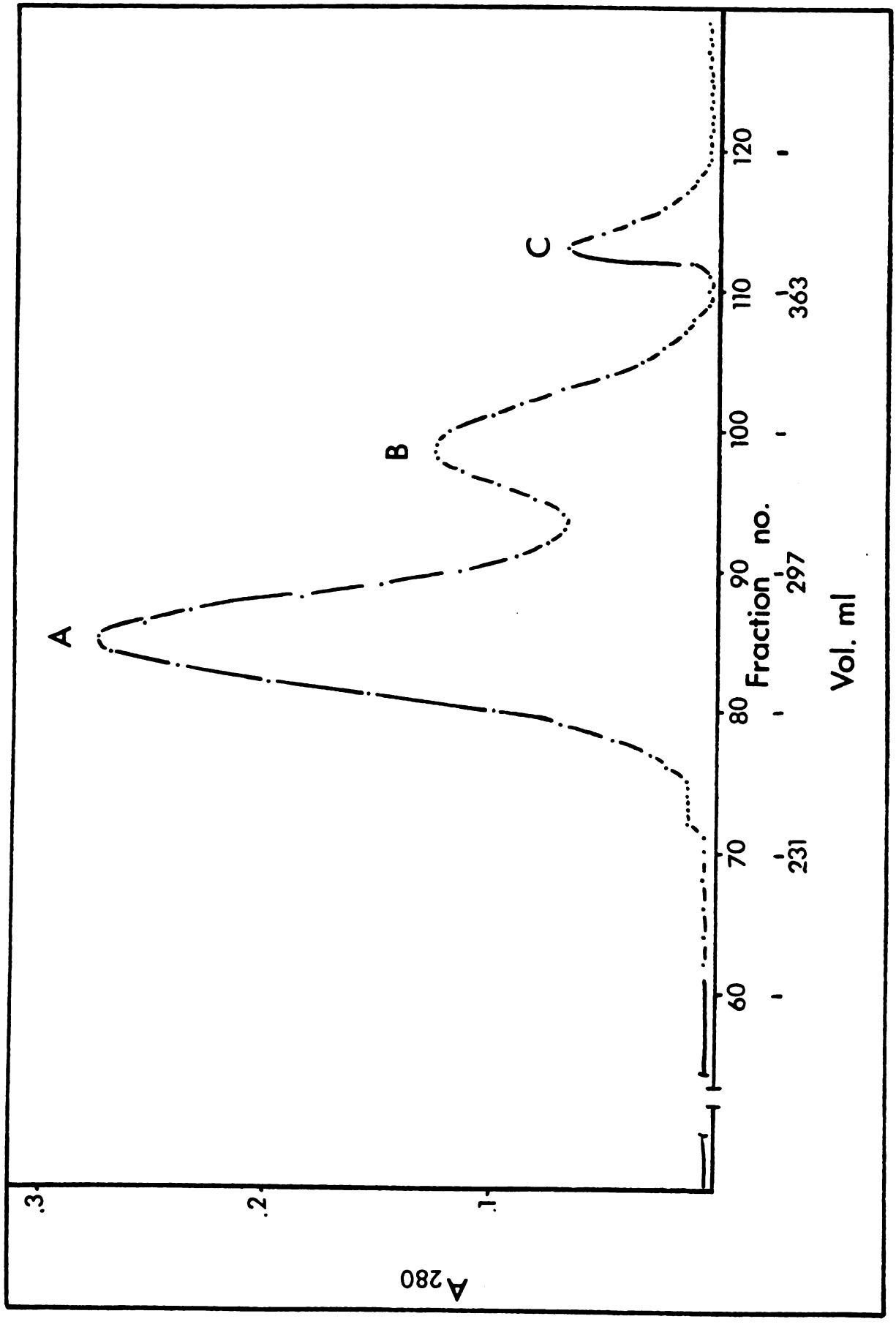
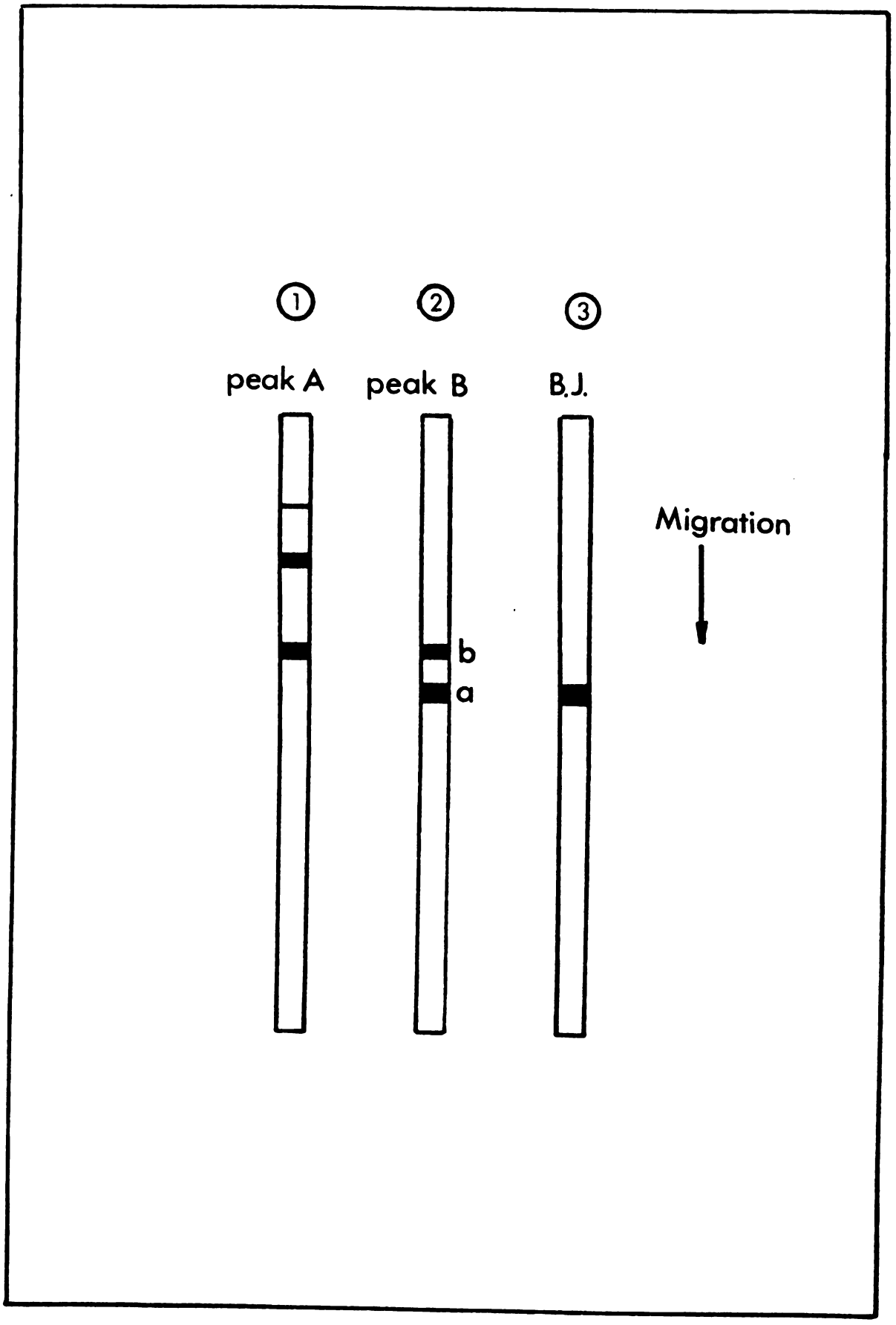

A280
.3
.2
.1
A
B
C
60
70
80
90
100
110
120
Fraction no.
231
297
363
Vol. ml

Figure 9.--SDS gel electrophoresis (10% acrylamide) for 5 1/2 hours at pH 7.0. Current 8 m A per tube.

Gel 1: Peak A from the gel chromatography of mixed heavy chains (IgG) and B. J. protein including all the non bound heavy chains.

Gel 2: Peak B from the gel chromatography of the hybridization solution. Peak includes the newly formed hybrids between heavy chains and B. J. protein.

Gel 3: B. J. protein (homogeneous as had been previously shown) was run as a standard for the migration of the light (B. J. protein) chains of the hybrids.


1
2
3
peak A
peak B
B.J.
b
a
Migration

has shown the same migration as the B. J. protein standard (light chain). And the (b) band has the same migration rate as the band of the heavy chain pattern (gel 1) with the higher migration (monomer heavy chain). The third gel is the standard: Light chains which had been used for the hybridization.

Fig. 10 is the gel filtration of the reduced but no alkylated heavy chains separated from their light chains. The first two peaks which correspond to polymer heavy chains and monomer heavy chains respectively are definitely separated from the peak of the light chains. The vertical lines indicate the fractions which were pooled and used for hybridization with B. J. proteins in order to make covalently bound hybrids. The volume of the pool was 62.6 ml and the sample concentration was .78 mg protein per ml (based on the absorbance at 280 mμ.).

After the addition of an excess of B. J. protein and dialysis of the mixture for 36 hours against sodium acetate buffer 0.02 M in 0.1 N NaCl, a gel Sephandex G-200 filtration was performed whose chromatography is shown in Fig. 11. Again the three peaks (heavy chains, hybrids and light chains) taken from the non-covalently bound hybrids filtration exhibit the same kind of filtration. This means that the results of the combination of reduced and carboxymethylated heavy chains with B. J. proteins and the results of the combination of the reduced but non alkylated heavy chains with B. J. proteins are similar. By the

Figure 10.--Gel chromatography of the mixture of heavy and light chains obtained from the reduction but none alkylated of IgG.

A G-100 2 cm x 95 cm column.

170 mg sample applied with 1 M propionic acid and 0.05 M thioglycerol to separate the heavy and light chains. Elution with 1 M propionic acid and 0.05 M thioglycerol. Flow rate = 5 ml per hour.

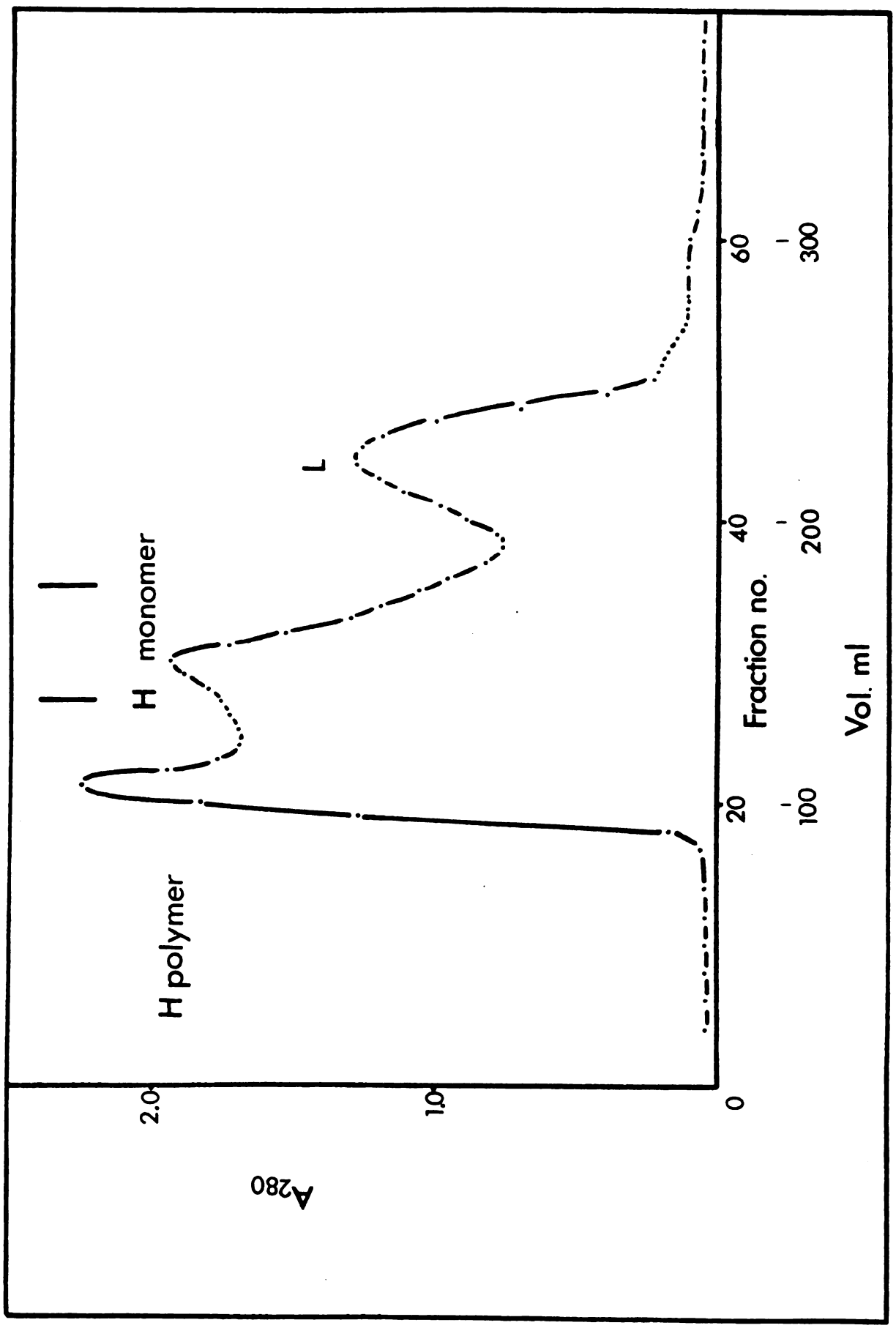
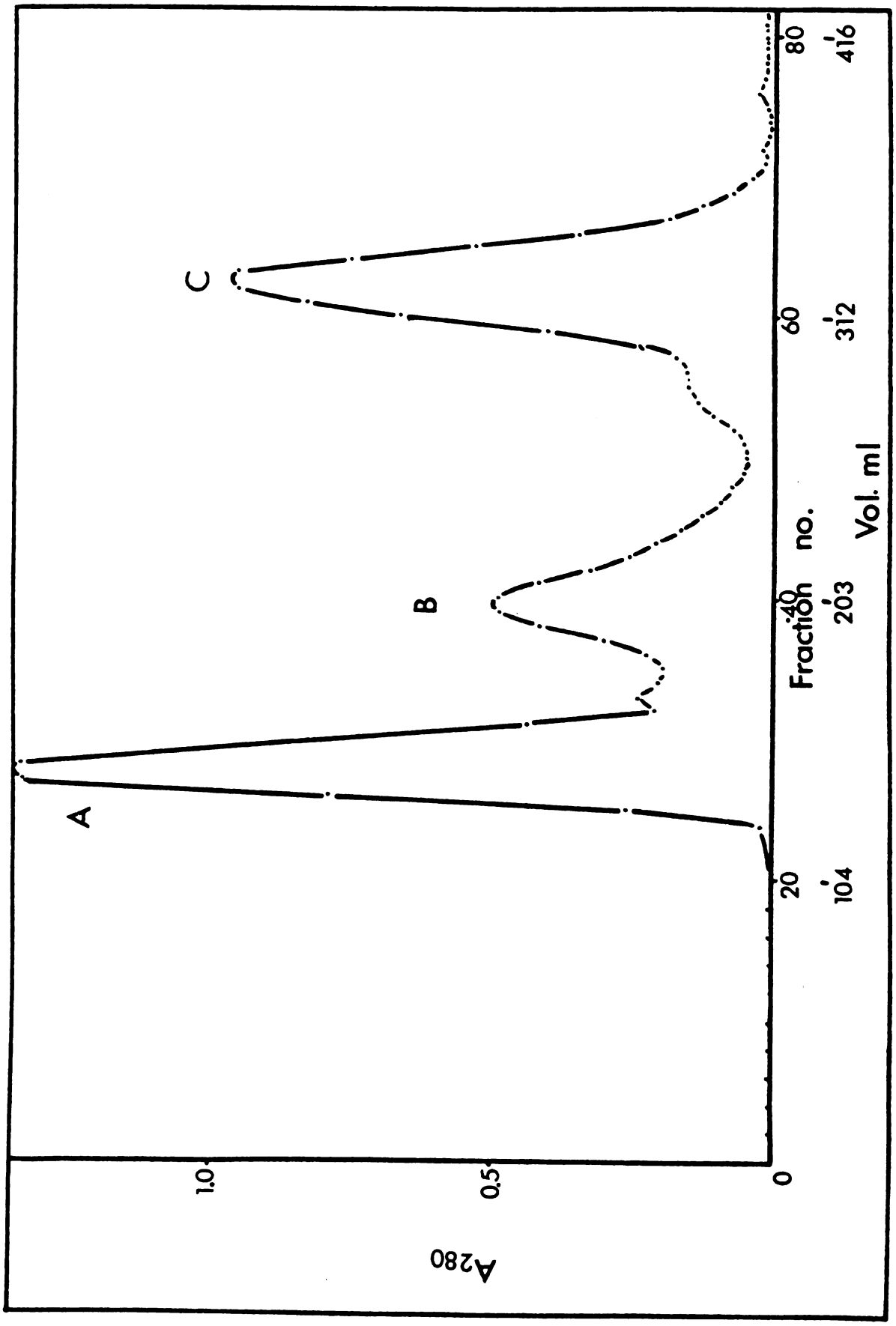

A280
2.0
1.0
0
H polymer
H monomer
L
20
40
60
Fraction no.
100
200
300
Vol. ml

Figure 11.--Gel chromatography on Sephadex G-200 of covalently combined heavy chains with B. J. protein. Column dimension = 2.5 cm x 95 cm, solvent 0.02 M sodium acetate pH 5.5 in 0.1 N NaCl 114.75 mg total protein (48.75 mg heavy chain plus 66 mg B. J. protein) in 13 ml of solvent applied 36 hours after mixing.

Peak A includes the heavy chains which have not been hybridized.

Peak B includes the covalently bound hybrids.

Peak C includes the non hybridized light chains (B. J. protein).


A280
1.0
0.5
0
A
B
C
20
40
60
80
104
203
312
416
Fraction no.
Vol. ml

simple mixing in 1:1 equimolar ratio of these two kinds of polypeptide chains or in excess of B. J. proteins hybrids can be formed which are noncovalently or covalently bound dependent upon the protection of the SH groups of the heavy chains after the reduction.

DISCUSSION

A number of studies have shown that mildly reduced and alkylated H and L chains from IgG molecules can be separated from each other with strong reagents, 1 M propionic acid, and then recombined upon renaturation in neutral pH (sodium acetate pH 5.5) or recombined in propionic acid, followed by dialysis to neutrality. This is true for chains derived from the same IgG molecule even if H and L chains are derived from two different animal species. Many biological, chemical and physical properties of the molecules derived from recombination have been studied which prove that these molecules regain their features and activities in a high percentage (75%-80%). It has been found that any pair of H and L chains form a stable IgG molecule but in the presence of autologous and heterologous L chains, a particular H chain combines with its autologous L chain to a higher degree.

In this work, even though the results are not quantitative, an idea of the degree of combination may be made by comparing our hybrid formations with that of recombined homologous chains of Tanford, it is possible to see that ours is low. It is because the L chain used (B. J. protein) is not autologous with any of the human

heavy chain mixture obtained from IgG molecules. The advantage of these hybrids is that they all contain the same light chain (homologous B. J. protein) such that they may be used for further studies in the immune response problem. These hybrids could serve as a model for the "pro-antibody" structure assumed in the hypothesis of the immune specificity being studied. For such molecular models, relatively stable molecules are needed, no pro-existing immune specificity and at least one of the two chains homogeneous.

In our case these characteristics were examined in the hybrids formed (non-covalently or covalently). First, the stability of these hybrids must not be low because of lengthy refrigerated storage in sodium acetate buffer pH 5.5. This prevents denaturation or precipitation and keep their absorbance at 280 M stable. Their denaturation was observed after lyophilysis, but this also is characteristic for many natural polypeptide molecules. Aggregation and precipitation of the hybrids was observed when they were concentrated after the dialysis of the mixture (H chains - B. J. protein) against sodium acetate buffer pH 5.5 in 0.1 N NaCl. This is why it was necessary to filtrate or to centrifuge the concentrated solution before hybrid filtration on the Sephadex G-200 column. To examine the precipitation problem more closely, the same procedure was repeated exactly for heavy chains only.

The same phenomenon of precipitation was observed in the concentration step showing that the heavy chains were responsible for precipitation in the hybrid mixture.

These hybrids, on the other hand, don't have any immune specificity since they consist of a mixture of heavy chains obtained from a mixture of human IgG molecules (fraction II Cohn) of different specificities. Their light chain is B. J. protein whose characteristic is the lack of any antigenic specificity.

Finally, these hybrids have a high degree of homogeneity since they contain the same light chains (the homogeneous B. J. protein).

Preliminary O.R.D. spectra of these hybrids are very similar to native IgG molecules. So hybrids may be used as a model of "pro-antibodies" whose difference from the specified antibody molecules includes small polypeptide regions due to transpeptidation.

Further outlined experiments include proteolysis of these hybrids with common digesting enzymes like trypsin, pepsin and chymotrypsin and amino acid analysis of the resulting peptides. Then immune reactions with a definite antigenic (eg. diptheria) will give specificity to these hybrids which will be digested in order to obtain peptides for comparison with those from the first proteolysis (before the immune reaction).

If this hypothesis could be proved, many questions about the immune response would be resolved: How can one organism have such a large number of antibodies specified for definite antigenes? How their amino acid sequences differ (variable regions) at the antigenic binding site? How an organism, which lacks a special antibody, after the antigenic infection can form a high number of these antibodies for the remainder of its life?

More studies have to be done on this area of research which is extremely interesting and is almost unknown.

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