IMMUNOCHEMICAL STUDIES OF MYOGLOBIN WITH SYNTHETIC PEPTIDES

Ph.D. CHEMISTRY

Joan Katherine Givas

IMMUNOCHEMICAL STUDIES OF MYOGLOBIN WITH SYNTHETIC PEPTIDES

ABSTRACT

The C-terminal heptapeptide of sperm-whale myoglobin and its C-terminal lower homologs, the hexa-, penta-, and tetra-peptides, as well as a tetradecapeptide, corresponding to residues 56-69 of myoglobin, were synthesized by the solid-phase technique. Immunochemical studies employing appropriate immunosorbents indicated that the N-terminal lysine residue of the heptapeptide played an important role in binding with rabbit antibodies directed to the heptapeptide. These antibodies were shown by electrophoresis in polyacrylamide gel to possess limited heterogeneity.

Attempts were made to measure the binding of (i) myoglobin, and (ii) N^{α} DNP-heptapeptide, with purified antibodies directed against the heptapeptide by the method of fluorescence quenching.

The antigenic activity of the synthetic tetradecapeptide was studied.

IMMUNOCHEMICAL STUDIES OF MYOGLOBIN WITH SYNTHETIC PEPTIDES

by

Joan Katherine Givas

A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Chemistry
McGill University
Montreal, Canada

July, 1971

To my Parents

ACKNOWLEDGEMENTS

I am grateful to Professor A.H. Sehon for his understanding and guidance during the course of this investigation and for his valuable help and criticism in preparing this thesis.

I am indebted to Dr. M. Manning for his assistance and the use of his facilities in synthesizing the peptides necessary for this study.

I wish to thank my colleagues for their advice and assistance during this study.

Finally, I am deeply grateful to my parents and friends without whose encouragement and help this study would not have been possible.

This investigation was supported by grants given to Professor A.H. Sehon by the National and Medical Research Councils of Canada, Ottawa, Ontario, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

General

When a material foreign to the circulation of an animal is introduced into the organism, it stimulates the production of proteins which combine specifically with it (1). The foreign substance is called an antigen and the proteins produced by the host in response to a given antigen are termed antibodies and are usually found in the serum of the animal. Formerly, it was thought that only proteins could be antigens but now many carbohydrates and lipids are known to be antigenic as well. Small molecules such as, for example, benzoic acid or nitrobenzene, designated as haptens, of themselves will not stimulate the production of antibodies; however, when coupled to larger molecules, e.g. albumin, they become antigenic (2,3).

The outstanding feature of antibodies is the high degree of their specificity in combining with the appropriate antigenic determinant group which enables them to distinguish between structural and even optical isomers of haptens (4,5).

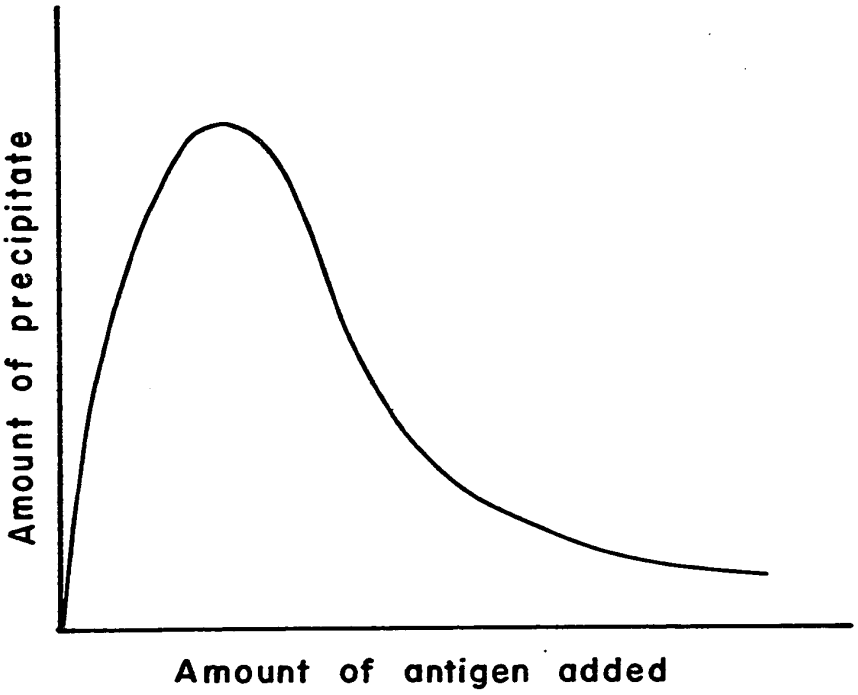
Detection of the Antigen-Antibody Reaction

(i) The precipitin reaction

When antibody is mixed with homologous antigen in vitro, and in the proper proportions, a precipitate occurs. Variation in the quantity of antigen added to an antiserum causes corresponding variation in the amount of precipitate produced. This can be expressed graphically by the classical precipitin curve (Figure 1). The features of this curve can be explained by

Figure 1

Typical precipitin curve.


Amount of precipitate
Amount of antigen added

the 'framework' or 'lattice' theory (6,7).

According to this theory, polyvalent antigen molecules combine with bivalent antibody molecules to form crosslinked aggregates thus making a larger lattice which eventually becomes a 3-dimensional framework and is rendered insoluble. This theory is based on the assumption that antibody and antigen molecules are multivalent, i.e., they have more than one combining site. In fact, for antigens this has actually been shown to be the case, and it has also been demonstrated that precipitating antibodies of the IgG class have a valence of two (8-10).

In the light of the lattice theory, ultracentrifugal and electrophoretic analyses have revealed that in the region of extreme antibody excess, small, soluble complexes consisting of one antigen and two antibody molecules exist. As the amount of antigen is increased, the region of maximal precipitation, referred to as the equivalence zone, is reached. As more antigen is added, the region of antigen excess or inhibition zone is attained. In this region, since there is much more antigen than is required to react with all the antibody sites, smaller antibody-antigen complexes are formed, and in large antigen excess soluble complexes consisting primarily of one antibody molecule and two antigen molecules are produced (11).

According to the foregoing explanation, it is evident that if haptens or univalent antigens are used, no precipitate can occur even in the equivalence zone. It follows that the precipitation of antibody-antigen complexes formed from multivalent reactants can be inhibited by the addition of hapten or

univalent antigen, or of univalent antibody fragments, to the antiserum prior to the addition of antigen. It also follows that if after formation of a precipitate, antigen is added, a disruption and loosening of the 3-dimensional lattice is effected with the formation of soluble complexes, and the precipitate begins to dissolve; this is predicated by a dynamic, reversible equilibrium for antibody-antigen reactions.

According to Kleczkowski (12), Najjar (13-15) and Sela (16), precipitation of antigen-antibody complexes can be explained on the assumption that flocculation and aggregation occur due to the interaction of hydrophobic regions of these molecules. Najjar proposes that some antibodies are 'subcomplementary' and that combination with antigen causes conformational changes which expose hydrophobic regions on the molecules. These newly exposed areas are then free to interact and aggregation results. Evidence for such changes was obtained by a study of the polarization of fluorescence of ovalbumin and anti-ovalbumin where conformational changes were observed in both antigen and antibody (17). Also Crumpton (18) has indicated an alteration of the conformation of metmyoglobin on combination with antibodies to apomyoglobin resulting in the release of heme.

The precipitin reaction may also be carried out in gels, in which the antigen and antibody are allowed to diffuse. One commonly used variation of this technique was developed by Ouchterlony (19). In this method, a layer of agar is allowed to solidify in a dish in which metal cylinders of an appropriate diameter are first placed. After the agar has solidified, the

cylinders are removed, thus leaving wells in the agar. The antigen and antibody are then placed in different holes and permitted to diffuse. When the two reagents meet each other there will be a narrow band at the position at which the relative concentrations permit a precipitate to form.

(ii) Hemagglutination

Agglutination tests are quite sensitive for detecting small amounts of antibody. In the passive agglutination tests, large particles, such as erythrocytes, are coated with the antigen, either by treating the cells with tannic acid (20) and then allowing the antigen to adsorb to the treated particle, or by coupling the antigen directly by means of a bifunctional reagent, i.e., bis-diazotized-benzidine (21). Addition of antibody to a suspension of antigen-coated red cells leads to the formation of a specific agglutination pattern due to the crosslinking of red cells into a gelatinous 3-dimensional network. This technique was shown to be at least 100 times as sensitive as the precipitin test (22).

(iii) Complement Fixation

This sensitive test is based on the fact that complement components, present in fresh serum of vertebrates, bind to antigen-antibody complexes, and on the fact that complement causes hemolysis of sheep erythrocytes which have been coated with rabbit antibody specific for sheep erythrocytes. This latter phenomenon may serve as a test for free complement. In the complement fixation test the antigen and antibody to be tested are allowed to react in the presence of complement.

When the sensitized sheep erythrocytes are added, the residual free complement causes hemolysis of the erythrocytes. The amount of hemoglobin thus released may then be measured spectrophotometrically. Hence, failure to obtain hemolysis indicates that sufficient antibody was present to bind all the complement, leaving none to hemolyze the indicator, sensitized sheep erythrocytes. Occurence of hemolysis indicates that insufficient antibody was present to bind all the complement thus leaving some free to hemolyze the red cells.

(iv) Phage-neutralization

When bacteriophage react with antibacteriophage antibodies the infectivity of the phage is neutralized; thus, by serial dilution of the phage (or antiserum) the amount of antibody present in the serum may be estimated (23). This method has been recently extended to include chemically modified bacteriophage (24-26). In this method an antigen or hapten is coupled to the phage and allowed to react with antibody specific for the conjugated antigen (or hapten). When the bacteria are added, it is found that the modified phage have been inactivated by the antibody, and the amount of antibody present may be quantitated by the degree of inactivation that occurs. (The visual aid is the number of plaques formed on the 'lawn' of bacteria.) The antibody may also be quantitated by measuring the degree of inhibition of inactivation. This is done by inhibiting the antigen-antibody reaction with the addition of free hapten to the antibody and modified phage. In this case, the antibody reacts with the free hapten, thus

preserving the infectivity of the phage. This method is extremely sensitive and it is estimated that antibody concentrations as low as 10^{-5} to 10^{-6} mg/ml and can be detected (27).

Immunogenicity and Antigenicity

In the light of evidence accumulated over the past few years, the concepts of immunogenicity and antigenicity are to be distinguished. The former may be defined as the ability of a molecule to elicit an antibody response (28). In contrast, the antigenicity or antigenic specificity may be employed to express the capacity of an antigen to react with antibodies, even if the antigen is deprived of the ability to provoke antibody formation. The reactive portion(s) of the molecule is sometimes referred to as an antigenic determinant or antigenic site, determinant group or simply as a hapten. A comparison may be drawn with enzymes where a certain area of the molecule may possess catalytic activity whereas a different site could be responsible for substrate binding. With antigens it is desirable to consider the moiety present in the molecule rendering it immunogenic as distinct from the portion responsible for its antigenic specificity.

For example, when gelatin, which is of itself a poor immunogen, was mildly tyrosylated (29), the antibody response was strongly augmented, but the antibody formed was specific for gelatin. In this case the tyrosine acted as an amplifier transforming the molecule into a better antigen without drastically changing its specificity.

Studies with synthetic polypeptides have shown that the

overall shape of the antigen does not seem to be a critical factor in immunogenicity since multichain polymers of different side-chain densities as well as linear polyamino acids may be immunogenic. The important factor in eliciting the biosynthesis of antibodies seems to be the accessibility of the immunogenically important area (30).

There does not seem to be a definite lower limit of molecular size or weight of an antigen to be able to induce an immune response. Until recently, the smallest compound capable to eliciting antibodies was a polypeptide of molecular weight 4000 (30). More recently, however, antibodies to low molecular weight substances have been reported. Thus, oxytocin (31) and vasopressin (32), both nonapeptides, were found to induce antibody formation in rabbits. Also, angiotensin, an octapeptide of molecular weight 1031, proved to be immunogenic in guinea pigs (33). In addition, N^{α} DNP*-hepta-L-lysine (mol.wt. 1080)(34), and p-azobenzene-arsonate-hexa-L-tyrosine (mol.wt. 1200)(35), as well as p-azobenzene-arsonate-N-acetyl-L-tyrosine and its D-analog (mol.wt. 451)(36), are immunogenic in guinea pig. However, although the hexa-tyrosine and hepta-lysine derivatives are immunogenic, a decapeptide, representing an antigenic determinant of tobacco mosaic virus protein (TMVP), and having about the same molecular weight as the former two peptides, was found to be non-immunogenic in guinea pigs (37). It would seem that the molecular weight required for immunogenicity

---

*DNP = 2,4-dinitrophenyl.

may vary from system to system and may be governed by the chemical nature of the determinant.

Early work in immunochemistry showed that antibodies are stereospecific, i.e., they distinguish between L- and D-optical isomers (2). However, until quite recently, only L-polymers were thought to be immunogenic. The work of Sela (38) and Gill (39) has shown that polymers composed exclusively of D-amino acids may be weakly immunogenic if administered in low doses. It is suggested that the weak antibody response to D-polymers is due to their slow and incomplete catabolism (40,41). Because of this, high doses of D-polymers are thought to cause paralysis of the immune system.

An inverse correlation between the net charge on the antigen and the type of antibody produced has also become evident* (42-45). The same hapten when coupled to proteins of different net charge give rise to different types of antibodies whose specificity is the same. These facts strongly support the suggestion that the biosynthesis of the antibody molecule is dependent not only on the antigenic determinant, but also on other areas of the molecule not involved in the definition of specificity. Nor is this effect limited to one species only, since human, rabbit, goat, horse and mouse sera behave in the same way.

The Nature of An Antigenic Determinant

Although the antigenicity of artificial and synthetic

---

*This aspect will be discussed on p. 37.

polypeptide antigens* (28,46-50) as well as of carbohydrates (51-53) has been widely studied, we are concerned here mainly with natural protein antigens.

For some time now it has been known that protein antigens possess a number of antigenic sites, the number probably increasing with increasing molecular weight. The number of antigenic determinants is not to be confused with the valence of the antigen, which represents the number of antibody molecules which the antigen can sterically accommodate. The number of antigenic sites may be far greater than the valence since some reactive sites may be buried inside the molecule, especially if the antigen is a globular protein.

The degradation of natural protein antigens has been tried by many investigators in an attempt to obtain fragments, representing single antigenic determinants, which could be used for elucidation of their structure. Cebra (54) digested silk fibroin, a fibrous protein, with chymotrypsin obtaining several peptides which were capable of inhibiting the precipitates formed by intact silk fibroin and the homologous antibodies. These peptides ranged from four to twelve amino acids in length. The inhibitory activity depended on the antiserum used and increased with increasing chain length, the most efficient inhibitor being a dodecapeptide. When the C-terminal tyrosine

---

*Artificial antigens may be defined as antigens obtained by chemical modification of natural antigens, e.g. poly-L-alanyl-bovine serum albumin. Synthetic antigens may be defined as antigens prepared by total synthesis, e.g. a copolymer of L-glutamic acid and L-tyrosine.

residue was removed from the octapeptide its activity decreased by one-half. Moreover, the molar ratio of inhibitor to antigen for 50% maximum inhibition was 5000. This is an extremely high value, indicating that the structure and/or size of the peptides differed greatly from that in the original molecule. From these results Cebra concluded that tyrosine in the antigenic determinant played an important part in the antigen-antibody reaction.

Several globular proteins have been degraded in an effort to obtain antigenic determinants, e.g., human serum albumin (HSA) (55,56), bovine serum albumin (BSA) (57), TMVP (58,59), diphtheria toxoid (60), fibrinogen (61), thyroglobulin (62), ribonuclease (RNAse) (63,64), lysozyme (65,66), staphylococcal nuclease (67), and myoglobin (68). The amino acid sequence of the last four of these proteins is known (69-73) and the complete 3-dimensional structure of RNAse (74), lysozyme (75) and sperm-whale myoglobin (76-80) have been fully elucidated by X-ray crystallography. The antigenicity of myoglobin will be discussed in detail in a separate section.

When TMVP was digested with trypsin it was found that an eicosapeptide (peptide # 8) bound specifically with rabbit antibodies directed to the whole protein (59,81). It was subsequently found that the C-terminal decapeptide (which was synthesized by the solid-phase method) was responsible for the antigenic activity of the eicosapeptide (82). Studies with the C-terminal lower homologs of this decapeptide revealed that the C-terminal acetyl-pentapeptide and all larger acetyl-

peptides were bound by anti-TMVP globulins. The smaller peptides were not effectively bound. As the chain length increased, the amount of peptide which was bound increased also. N-octanoylation of the C-terminal tripeptide produced strong binding with antibody indicating that the hydrophobicity conferred by octanoylation was responsible for the enhanced binding (83). Several authors have suggested that antibody combining sites directed against haptens may be hydrophobic in nature (84,85), a suggestion which may prove at least partially true for antibodies directed against proteins. It should be pointed out that anti-TMVP sera from some rabbits showed no binding to the decapeptide or lower homologs while sera from other rabbits showed binding; all sera showed binding with the eicosapeptide. These facts point to the heterogeneity of the antibody response even to a small antigenic area.

Lysozyme was digested with pepsin (86,87) and two independent antigenic sites were isolated (88), one of which contained the 'loop' region, so-called since it forms a loop bound by a disulfide bond. A loop-peptide of lysozyme, containing 19 amino acids, was isolated by Arnon (89) and antibodies to this unique region were isolated which did not precipitate with lysozyme, indicating that they were specific for only one region of lysozyme. Moreover, a synthetic conjugate, prepared by covalent binding of the loop-peptide to a synthetic branched polypeptide, elicited antibodies in rabbits with specificity directed against this region of native lysozyme (66).

Studies with staphylococcal nuclease have indicated that

this molecule possesses at least two antigenic determinants which may be localized to the linear sequences, residues 18-47 and 127-149. In addition to these determinants, immunosorption and binding studies indicate the possibility of other determinants which are formed by the conformation of the native protein (67,90).

It must be pointed out that in contrast to fibrous proteins, globular proteins have a more rigid secondary and tertiary structure permitting amino acids which are far apart in the extended polypeptide chain to assume contiguous positions in the folded chain. Hence, it may well be that non-sequential portions of a polypeptide are physically adjacent in the 3-dimensional native conformation of the protein and constitute the antigenic site(s), but when the antigen is degraded the recovered active peptides represent only portions of the site whose amino acids are sequential. This is perhaps one of the most important factors to be considered in the degradation of protein antigens. The importance of the role of the secondary and tertiary structures in antigenicity is demonstrated by studies with RNAse (91), where breakage of the disulfide bonds resulted in complete loss of antigenic activity. Such conformation-dependence of antibodies is also evident in the lysozyme-antilysozyme system where carboxymethylation and performic acid oxidation of the loop-peptide resulted in a drastic decrease in the reactivity of this peptide with loop-specific antibodies (92). Also, loss of the immunological activity of hemoglobin A resulted from separation of the α and β chains (93). It has also been shown that a polymer of the tripeptide,

Tyr-Ala-Glu, which exists as an α-helix under physiological conditions, and the same tripeptide when attached to a branched polymer, elicit antibodies which do not cross react (94). Moreover, the tripeptide is an efficient inhibitor of the antigen-antibody reaction in the case of the branched polymer but no inhibition is observed with this tripeptide with the α-helical antigen. In addition, studies with artificial myoglobins in which the metalloporphyrin, which is not a part of an antigenic site (95), was modified, indicated that the observed changes in antigenic reactivity could be attributed to conformational reorganization caused by the different coordination tendencies of the various metals used in the porphyrin, or to modification of the side-chains of the heme group (96,97).

From these studies, two types of antigenic determinants can be distinguished (98). Sequential determinants are due to the amino acid sequence in a random coil form and antibodies to this type of determinant should react with peptides of identical or similar sequence. On the other hand, conformational determinants result from the 3-dimensional conformation of the antigen and lead to antibodies which might not necessarily react with peptides derived from that area of the molecule or with the antigen whose original steric conformation has been changed.

With regard to the size of an antigenic determinant, the more active peptides isolated from the degraded proteins mentioned above ranged from a molecular weight of 665 (six amino acids) for myoglobin to a molecular weight of about 15,000 for HSA (55). It is significant that in general the molar ratio

of inhibitor to antigen giving 50% maximum inhibition decreased with increasing chain length. Also, two different myoglobin peptides of different chain lengths (19 and 15 amino acids), representing the same antigenic site and giving the same degree of inhibition (12%) had different molar ratios of inhibitor to antigen, i.e. 12 and 50 respectively (68). This suggests that inhibitory capacity may not reflect the 'completeness' of the antigenic site, but may be an indication of similarity of conformation between the isolated peptide and the original native antigen. However, it should be pointed out that in this case, the four residues, making the difference between the two peptides, were hydrophobic in nature, and it is possible that some non-specific hydrophobic interaction may have occurred with the larger peptide, as was the case with studies involving TMVP (83).

Studies by Kabat (51), employing inhibition of precipitation of the dextran-antidextran system, showed that in this system the size of the antigenic site was a hexasaccharide, ismalto-hexaose, having molecular weight 990 and measuring in its most extended form 34 x 12 x 7 Å. Isomaltoheptaose was not a better inhibitor on a molecular basis. This value is in agreement with that obtained by Sage (99) for a penta-alanine peptide, 25 x 11 x 6.5 Å. Studies with other artificial and synthetic antigens (100,101) also show that antibodies were inhibited maximally with pentapeptides. Investigations with polyalanyl determinants containing L- and D- residues in strategic positions indicate that the size of an antigenic site is a tetra-alanine (102,105).

Of special interest are the studies with N^{α} DNP-oligolysines of different chain lengths (106). It was found that, on a molar basis, maximum inhibition of precipitation of guinea pig anti-DNP $(Lys)_{11}$ sera was obtained with the heptalysine derivative. The octa- and nona- peptides were no better inhibitors than the heptamer, suggesting that the upper limit of the size of the combining site in the N^{α} DNP$(Lys)_n$ system is complementary to the heptamer, which in its most extended form measures 30 x 17 x 6.5 Å (107). In contrast, when N^{α} DNP$(Lys)_{60-1200}$ were used as the immunizing antigens, antisera to these polymers were maximally inhibited by N^{α} DNP$(Lys)_3$. Hence, these results indicate that the chain length and conformation of the antigen affect the specificity of the antibody formed to DNP-polylysines (108).

In a separate study (100), it was found that a penta-D-Lysine peptide proved to be the most effective inhibitor of the rabbit anti-poly-D-Lysine and poly-D-Lysine system, as measured by complement fixation. The reason for the difference between these results and the former results is not understood; however, different materials for immunization, as well as different methods of measuring inhibition were used, and in addition, the antibodies were elicited in different species.

Structure and Chemistry of Myoglobin

Myoglobin is a globular protein containing one heme group and consisting of one polypeptide chain. Physiologically, it is found in muscle cells where it serves to store molecular oxygen until the metabolic processes of the cells require it.

Myoglobin consists of 153 amino acid residues (Figure 2, Table I) on the basis of which the molecular weight is calculated to be 17,816 (109).

The prosthetic group of myoglobin is a very stable coordination compound of iron with protoporphyrin IX (110). In heme, the ferrous atom at the centre of the porphyrin ring has four of its six coordination sites occupied by bonds with the porphyrin. The fifth and sixth free coordination positions are available for combination with ligands, one on each side of the plane of the porphyrin. The chief physiological function of myoglobin, the reversible binding of molecular oxygen, occurs at one of the free coordination sites of the heme iron atom, the other site being occupied by a water molecule (111). In order to bind and release oxygen, the iron atom must be in the ferro state. Heme which is not bound to apomyoglobin (a name referring to the residual protein after removal of the heme) is rapidly oxidized to the ferri state, rendering it useless. Thus the protein portion of metmyoglobin (a name denoting the protein-porphyrin complex) serves to protect the environment of the heme ensuring its proper functioning.

In the myoglobin molecule, almost all the polar side-chain groups are on the surface or protruding into the solvent. Two exceptions to this are the histidine residues (E7 and F8) in contact with the heme group. The molecule contains a relatively large number of non-polar residues; these make up about 42% of the total amino acids and are, with a few exceptions, almost all in the 'interior' of the molecule. It is evident that

Figure 2

Alpha-carbon diagram of myoglobin. Large dots represent α-carbon positions. For identification of residues, see TABLE I. The labeling is that of Kendrew et al. (80). Stretches of α-helix are represented by the smooth curved lines (except for the D-helix) and non-helical regions are represented by straight lines between atoms (zig-zag appearance). Fainter parallel lines outline the high density region as revealed by the 6-Å analysis. Heme group is toward top center with side chains identified by: M = methyl, V = vinyl, P = propionic acid. Five-membered rings at F-8 and E-7 represent the imidazole rings of the histidine residues associated with the heme.

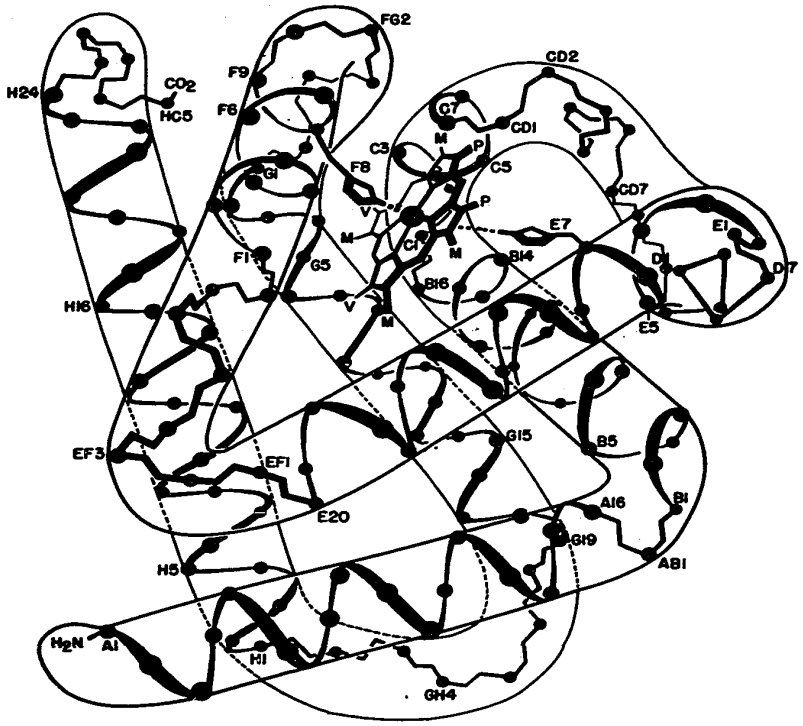

FG2
CD2
H24
CO_2
HC5
F9
F6
C7
M
CD1
C3
M
P
C5
F8
G1
CD7
V
P
E7
E1
M
C1
M
B14
F1
G5
D1
D7
B16
H16
V
M
E5
G15
B5
EF3
EF1
A16
B1
E20
G19
H5
AB1
H_2N
A1
H1
GH4

TABLE I

Amino acid sequence for sperm-whale myoglobin. The number column to the left is the number of the residue in the total sequence and the center column gives the position of the residue in its helical or non-helical region. Helix regions are A, B, C, D, E, F, G, and H, while non-helical regions are NA, AB, CD, EF, FG, GH, and HC (See Figure 2).

TABLE I

1	NA-1	Val	52	D-2	Glu	103	G-4	Tyr
2	2	Leu	53	3	Ala	104	5	Leu
3	A-1	Ser	54	4	Glu	105	6	Glu
4	2	Glu	55	5	Met	106	7	Phe
5	3	Gly	56	6	Lys	107	8	Ile
6	4	Glu	57	7	Ala	108	9	Ser
7	5	Trp	58	E-1	Ser	109	10	Glu
8	6	Gln	59	2	Glu	110	11	Ala
9	7	Leu	60	3	Asp	111	12	Ile
10	8	Val	61	4	Leu	112	13	Ile
11	9	Leu	62	5	Lys	113	14	His
12	10	His	63	6	Lys	114	15	Val
13	11	Val	64	7	His	115	16	Leu
14	12	Trp	65	8	Gly	116	17	His
15	13	Ala	66	9	Val	117	18	Ser
16	14	Lys	67	10	Thr	118	19	Arg
17	15	Val	68	11	Val	119	GH-1	His
18	16	Glu	69	12	Leu	120	2	Pro
19	AB-1	Ala	70	13	Thr	121	3	Gly
20	B-1	Asp	71	14	Ala	122	4	Asn
21	2	Val	72	15	Leu	123	5	Phe
22	3	Ala	73	16	Gly	124	6	Gly
23	4	Gly	74	17	Ala	125	H-1	Ala
24	5	His	75	18	Ile	126	2	Asp
25	6	Gly	76	19	Leu	127	3	Ala
26	7	Gln	77	20	Lys	128	4	Gln
27	8	Asp	78	EF-1	Lys	129	5	Gly
28	9	Ile	79	2	Lys	130	6	Ala
29	10	Leu	80	3	Gly	131	7	Met
30	11	Ile	81	4	His	132	8	Asn
31	12	Arg	82	5	His	133	9	Lys
32	13	Leu	83	6	Glu	134	10	Ala
33	14	Phe	84	7	Ala	135	11	Leu
34	15	Lys	85	8	Glu	136	12	Glu
35	16	Ser	86	F-1	Leu	137	13	Leu
36	C-1	His	87	2	Lys	138	14	Phe
37	2	Pro	88	3	Pro	139	15	Arg
38	3	Glu	89	4	Leu	140	16	Lys
39	4	Thr	90	5	Ala	141	17	Asp
40	5	Leu	91	6	Gln	142	18	Ile
41	6	Glu	92	7	Ser	143	19	Ala
42	7	Lys	93	8	His	144	20	Ala
43	CD-1	Phe	94	9	Ala	145	21	Lys
44	2	Asp	95	FG-1	Thr	146	22	Tyr
45	3	Arg	96	2	Lys	147	23	Lys
46	4	Phe	97	3	His	148	24	Glu
47	5	Lys	98	4	Lys	149	HC-1	Leu
48	6	His	99	5	Ile	150	2	Gly
49	7	Leu	100	G-1	Pro	151	3	Tyr
50	8	Lys	101	2	Ile	152	4	Gln
51	D-1	Thr	102	3	Lys	153	5	Gly

hydrophobic bonding plays a major role in stablizing the conformation of this protein.

Metmyoglobin appears to be quite stable over a wide pH range, i.e. from about pH 4.5 to pH 10 (112). Below pH 4.5 conformational changes take place very rapidly and are quite extensive as evidenced by changes in viscosity.

Removal of the heme group from myoglobin (effected at about pH 1.5) seems to produce some changes in the conformation of the polypeptide chain. Studies of the side-chain reactivities (113), α-helix content by optical rotatory dispersion and circular dichroism measurements (114,115), hydrodynamic measurements (116), and immunological activities (95,18) of the metmyoglobin and apomyoglobin molecules, reveal significant differences between the two proteins. Further, apomyoglobin is degraded very readily by the action of trypsin and chymotrypsin, but native metmyoglobin almost completely resists the attack of these enzymes (68). The fact that the heme can be dissociated from metmyoglobin and reassociated with a complete recovery of the native conformation of the conjugated protein indicates that interaction of the heme with the protein is necessary for the stabilization of the conformation of metmyoglobin.

Antigenicity of Myoglobin

Sperm-whale myoglobin is one of the best antigenically characterized proteins known. Tryptic (117) and chymotryptic (68) digests of apomyoglobin were obtained and the individual peptides isolated and tested for immunologic activity by measuring the inhibition of precipitation of anti-metmyoglobin sera with

metmyoglobin or apomyoglobin. Cyanogen bromide cleavage products were also obtained and the resulting peptides tested in the same way. A summary of the antigenic regions, their comparative amino acid sequences, and their inhibitory activities is given in Tables II and III.

These peptides range in length from six to twenty-four amino acids. All of the active sites, with the exception of peptide 70-76, contain a large number of polar residues, and only four of them, i.e., peptides 15-33, 119-133, 139-146 and 147-153, contain aromatic side-chains. It is worth noting that acetylation of the free amino groups of myoglobin resulted in total loss of precipitating activity (118) indicating that the polar amino groups play a vital role in antigen-antibody reactions with myoglobin. However, the possibility of configurational change due to acetylation cannot be ruled out.

The amino terminal heptapeptide in helix A (Figure 2), which was isolated from the tryptic and chymotryptic digests, did not possess any inhibitory activity (68-117). Also, the activity of myoglobin, which had been modified at tryptophan-7 (119), was the same as that of the unmodified protein. Moreover, cleavage at this residue with periodate (120) resulted in a shortened protein which retained the activity of native myoglobin.

The region consisting of residues 15-33, part of which constitutes the corner between helices A and B, probably contains only one antigenic determinant since it was found that peptides 15-33 and 15-29, individually or when mixed together, resulted

TABLE II

Antigenically active peptides of Sperm-Whale myoglobin.

TABLE II

Chymotryptic Peptides (68)				Tryptic Peptides (117)			
Sequence	3-D Model	% Inhibition*		Sequence	3-D Model	% Inhibition*	
		Mb	Apo-Mb			Mb	Apo-Mb
15-29	A13-B10		12	17-31	A15-B12	6	11
15-33	A13-B14		12				
56-69	D6 -E12	8	0				
70-76	E13-E19		7				
77-89	E20-F4		9	79-96	EF2-FG2	6	11
				119-133	GH1-H9	5	
139-146	H15-H22		5				
132-153**	H8 -HC5	8	19-30	132-153**	H8 -HC5	21	18.5
147-153	H23-HC5	7	15	148-153	H24-HC5	6	11

*Inhibitory activity of peptides was measured using anti-metmyoglobin sera.

**Obtained by cyanogen bromide cleavage.

TABLE III

Sequences of antigenically active peptides of myoglobin.

TABLE III

```
15                  20                  25                  30
Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg*-Leu-Phe
Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu
       Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg

56              60                  65
Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly-Val-Thr-Val-Leu

70                  75
Thr-Ala-Leu-Gly-Ala-Ile-Leu

77          80                  85                  90                  95
Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu
       Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys

119                 125                 130
His-Pro-Gly-Asn-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-Met*-Asn-Lys

            135                 140                 145                 150
Asn-Lys-Ala-Leu-Glu-Leu-Phe-Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr+-Lys-Glu-Leu-Gly-Tyr+-Glu-Gly
                          Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr+
                                                    Lys-Glu-Leu-Gly-Tyr+-Glu-Gly
                                                        Glu-Leu-Gly-Tyr+-Glu-Gly
```

*Found to be unnecessary for antigenic reactivity.

+One or both tyrosines 146 and 151 are necessary for antigenic reactivity.

in the identical maximum inhibition (12%) of the precipitation of apomyoglobin (68). However, the molar ratio of inhibitor/antigen required for 50% maximum inhibition was only 12 for the longer peptide as compared with 50 for the shorter one. This may be due to a closer approximation of the longer peptide to the original configuration in the native molecule, although hydrophobic interaction of the four additional residues cannot be ignored (83). Peptide 17-31 showed about the same inhibitory activity (11% with apomyoglobin) but in this case the molar ratio of inhibitor/antigen for 50% maximum inhibition was only 11. The reason for the difference between this peptide and the other two in the same region is not known, but throughout these studies different sera behaved in different manners toward the same peptides. Moreover, arginines 31 and 45 were shown to be unessential for antigenic activity since modification of these residues with cyclohexanedione did not change the immunochemical reactivity of peptide 1-55 (121).

The region between arginines 31 and 45 was also inactive (117) and oxidation of methionines 55 and 131 with periodate resulted in a modified myoglobin which was immunochemically indistinguishable from native myoglobin, showing that these two methionine residues were not essential to the antigenic activity of myoglobin (122).

Peptide 56-69 inhibited complex formation only with metmyoglobin and to the extent of 8% (68). This peptide is helical in the native molecule and contains the E7 histidine residue (Figure 2, Table I) hydrogen bonded to a water molecule which is coordinated to the heme-iron atom. It is quite

possible that upon removal of the prosthetic group this region assumes a different conformation. Although this peptide contains little or no helical conformation in aqueous solution (123) it still combines with antibody, although it requires a very high molar ratio of inhibitor/antigen for 50% inhibition, e.g., 145, which may indicate some dependence of the antibodies on conformation. In fact this is true of almost all the peptides isolated. Circular dichroic and optical rotatory dispersion measurements have shown that even relatively long segments of the polypeptide chain have a much reduced helical content compared with the same peptide in the native protein (124-125).

Peptide 70-76 is also antigenically active, inhibiting precipitation with apomyoglobin to the extent of 7%. This peptide is mainly hydrophobic in nature and it is significant that it is active since it constitutes a small portion of the E-helix.

The next active region along the polypeptide chain is represented by peptides 77-89 and 79-96, which constitute the bend EF, the helical region F, and a part of the corner FG (Figure 2). Peptide 77-89 inhibits precipitation with apomyoglobin (9%) while peptide 79-96 inhibits precipitation with both apomyoglobin (11%) and metmyoglobin (6%).

It is noteworthy that the last three antigenically active regions (residues 56-96) are contiguous along the polypeptide chain and include two helices and three corners (Figure 2). This may be suggestive of a special role played by more accessible

corners or bends in a protein antigen.

Tyrosine-103 was found to be unnecessary for antigenic activity (127), but arginine-118 was shown to be located in an active region since its modification resulted in an appreciable decrease of the immunochemical reactivity of cyanogen bromide peptide 56-131 (121). Furthermore, a peptide obtained by cleavage at proline residues (126) consisting of residues 37-87, had appreciably less inhibitory activity than an overlapping peptide, 37-119, implicating another reactive region in the longer peptide, probably around arginine-118. In addition, peptide 119-133, which is made up of the corner region GH and a part of helix H, inhibited precipitation with metmyoglobin to the extent of 5%. In all probability, the residues near, and including, arginine-118 along with a portion of peptide 119-133 form a part of the same antigenic region. Methionine-131 does not form a part of the active site (122), hence, it is likely that the reactive region terminates before this residue.

Peptide 132-153, which constitutes the C-terminal 22-amino acids and includes two small reactive peptides, is largely helical in nature in the native molecule. Both methionine-131 and arginine-139 do not contribute to the antigenic activity, hence, the N-terminal octapeptide of peptide 132-153 is probably immunochemically inactive (121,128). The inhibitory activity of peptide 139-146 is questionable since in one study (68) it was found to inhibit apomyoglobin marginally (5%), while in another study it was shown to have little or no inhibitory activity (128).

The C-terminal heptapeptide, comprising residues 147-153, and the C-terminal hexapeptide have been the object of many investigations (68,117,129,130). The heptapeptide, which is essentially non-helical in the natural protein, inhibits the precipitation of apomyoglobin up to 15%. Upon further treatment with chymotrypsin, which removes the C-terminal dipeptide, the activity of the heptapeptide decreased by more than one -half (68,128). These results are strikingly different from those reported by Atassi (118), who found that after removal of the C-terminal dipeptide of myoglobin the precipitin activity of myoglobin was not diminished. These observations may be reconciled if the dipeptide serves to orientate the heptapeptide into a more favorable conformation for binding with antibody. The dipeptide may not be needed for such orientation in the native molecule (131). In studies with natural and synthetic C-terminal fragments of the heptapeptide (130) it was found that the hepta- and hexa- peptides did not differ in their ability to inhibit the precipitation of apomyoglobin, and the penta- and tetra- peptides possessed much less inhibitory activity than the hepta- and hexa- peptides. Furthermore, replacement of tyrosine-151 in the hepta-, hexa-, and penta-peptides by phenylalanine or p-methoxyphenylalanine resulted in no significant change in activity. In contrast, nitration of tyrosines 146 and 151 of peptide 132-153 completely destroyed the inhibitory activity of this peptide (127). From the foregoing discussion, it is clear that the precise delineation of this antigenically reactive region must await further study.

It should be emphasized that, in these studies, as pointed out earlier, not all the antisera behaved in the same way with the same peptides, i.e., some peptides showed inhibitory activity with only some antisera, pointing again to the heterogeneity of the antibody response even to a single, relatively small area of the antigen.

Although the crystalline structure of myoglobin has been elucidated it must be pointed out that a study of the antigenicity of myoglobin involves its reactions in solution. Investigations of the correlation between the crystalline and soluble structures of myoglobin indicate that the structures of the molecule in the two states are in very close agreement. Hydrodynamic measurements (132,133,116) indicate that the molecule in solution has a very compact structure and that its dimensions are fairly close to those of the molecule in the crystalline state. The helix content of the myoglobin molecule in solution, determined by optical rotatory dispersion and circular dichroism measurements, corresponds to the 75% helix content observed in the crystalline structure (134,135).

Antibody Structure

There are five known classes of human immunoglobulins, IgG, IgA, IgM, IgD and IgE, whose molecular weights range from 150,000 to 1,000,000 and whose carbohydrate contents vary from 2.5% to 12%. In man and rabbit, the bulk of antibody activity appears to be associated with the IgG and IgM classes. IgA is present in higher concentrations in secretions. Reaginic antibody, i.e., skin-sensitizing antibody produced by allergic

individuals is associated with the IgE class (136). The general properties of these five classes of immunoglobulins are summarized in Table IV (137). So far, only IgG antibodies (comprising about 85% of the total immunoglobulins) have been investigated in detail. Recently, the entire covalent structure of a human IgG myeloma protein has been determined (138).

The subject of immunoglobulins is a vast and complex one, and hence, we will be concerned here mainly with rabbit IgG. Porter (139) postulated a symmetrical, four chain structure for rabbit IgG immunoglobulins which has subsequently been found characteristic for all mammalian species so far investigated (Figure 3). These polypeptide chains are held together by disulfide linkages. Two chains are referred to as heavy chains (mol.wt. 50,000) and two are termed light chains (mol.wt. 20,000) (140,141). Porter's model is based on his evidence obtained from the digestion of rabbit IgG with papain (142) and on data obtained from the separation of the polypeptide chains by Edelman and Poulik (143).

Papain cleaves IgG into three fragments, two Fab and one Fc (Figure 3). The Fab fragments each have a molecular weight of 52,000 and each one carries one antibody combining site. The Fc fragment has a molecular weight of about 48,000, possesses no antibody activity and is crystallizable. In the absence of a reducing agent, pepsin cleaves IgG into a divalent fragment, $F(ab')_2$, which has a molecular weight of about 100,000; the Fc fragment is degraded into smaller peptides. The $F(ab')_2$ fragment can be split into two equal halves by reduction of a single disulfide bond with thiol, each Fab' half being univalent

TABLE IV

Classes of human immunoglobulins.

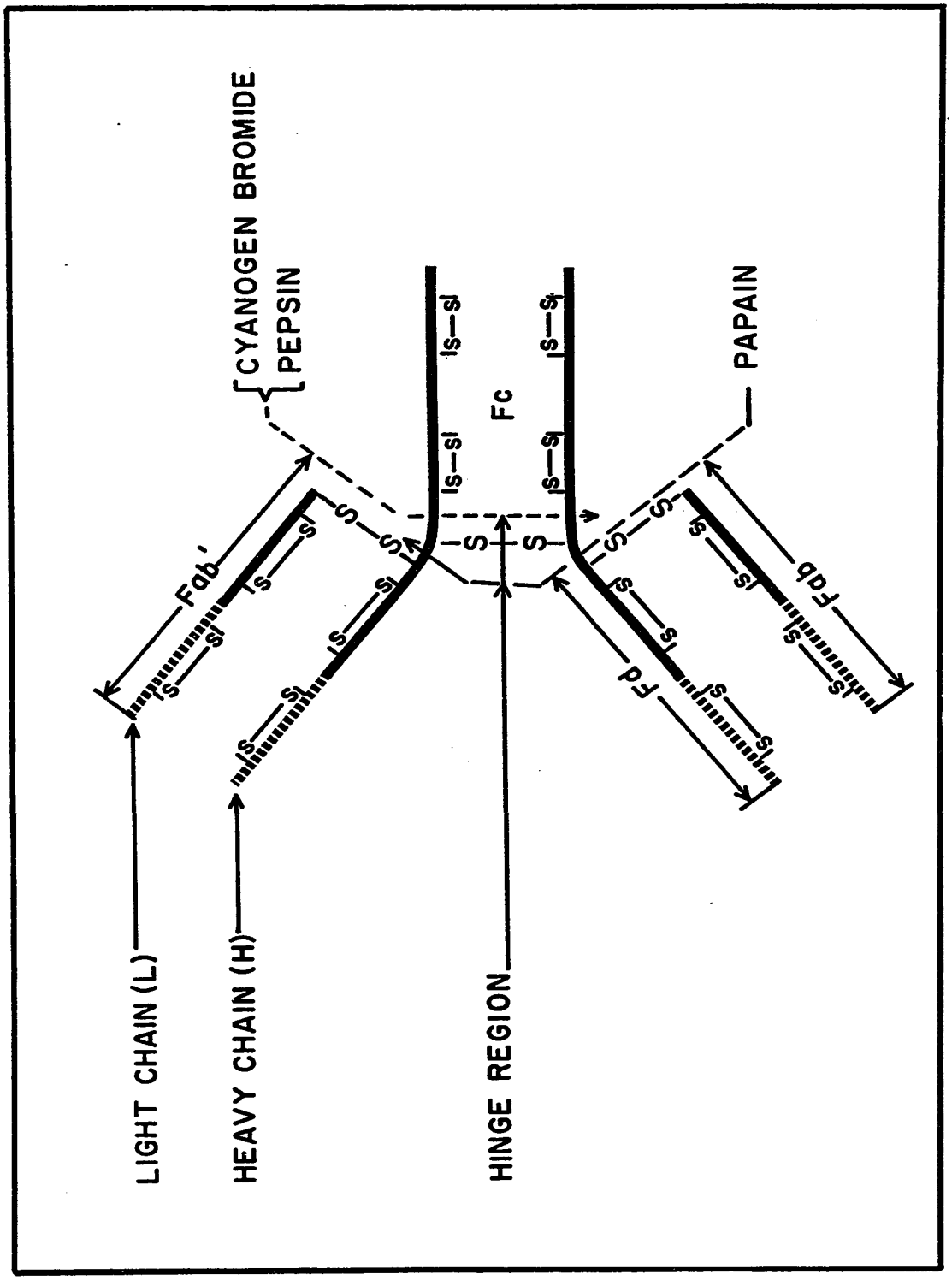
TABLE IV

Classes of Human Immunoglobulins

Class	γG	γA	γM	γD	γE
Heavy Chains:					
class	γ	γ	μ	δ	ε
subclasses	γ1,γ2,γ3,γ4	α1,α2		—	
mol. wt.	53,000	64,000	70,000	—	75,000
Light Chains:	κ,λ	κ,λ	κ,λ	κ,λ	κ,λ
mol. wt.	22,500	22,500	22,500	22,500	22,500
Mol. formula	$(\kappa_2\gamma_2)$ or $(\lambda_2\gamma_2)$	$(\kappa_2\alpha_2)_n$ or $(\lambda_2\alpha_2)_n$ n = 1,2,3 ...	$(\kappa_2\mu_2)_5$ or $(\lambda_2\mu_2)_5$	$(\kappa_2\delta_2)$ or $(\lambda_2\delta_2)$	$(\kappa_2\varepsilon_2)$ or $(\lambda_2\varepsilon_2)$
$S_{20,w}$	6.5-7.0	7,10,13,15	18-20	6.2-6.8	7.9
Mol. Wt.	150,000	180,000-500,000	950,000	—	196,000
Carbohydrate (%)	2.9	7.5	11.8	—	10.7

Figure 3

Schematic structure of the IgG molecule. The various positions of cleavage and the resulting subunits are indicated.


CYANOGEN BROMIDE
PEPSIN
PAPAIN
Fab'
Fc
Fab
Fd
LIGHT CHAIN (L)
HEAVY CHAIN (H)
HINGE REGION

and similar to Porter's Fab fragment (144,145). Cleavage of IgG with cyanogen bromide closely resembles peptic digestion. A major divalent fragment is produced similar to $F(ab')_2$ and can be reduced in the same way to two univalent fragments (146). The various positions of cleavage are shown diagrammatically in Figure 3. It seems that a relatively small area of the molecule (hinge region) is susceptible to cleavage.

Treatment of IgG with 2-mercaptoethanol followed by gel filtration resulted in a separation of the heavy and light chains (143). Each light chain is connected by a single disulfide bond, and the heavy chains are connected to each other by one or more disulfide bonds, depending on the subclass (147,148). Studies of the chemical structure of both heavy and light chains revealed that the amino acid sequence in the C-terminal end of all chains, consisting of approximately one-half of the chain, was constant; the N-terminal end, which constitutes the portion carrying antibody activity, was variable (149-151).

It should be mentioned here that IgM is a pentamer consisting of five 7S subunits, linked together by single disulfide bonds. Each 7S subunit is similar to the general structure of IgG, containing two heavy and two light chains joined by disulfide bonds (152). Fragments may be obtained from IgM subunits which are analogous to Fab and Fc fragments from IgG (153,154).

A more detailed description of the structure and properties of antibodies can be found in review articles by Cohen and

Porter (155), Haurowitz (156), Fleischman (157), and Edelman and Gall (137).

Overall Shape of Immunoglobulins

Electron microscopy studies have suggested that IgG is an elongated molecule having a length of about 250 Å and a diameter of about 30-40 Å (158). In addition, IgG antibody, when linked with divalent hapten, was shown to form Y-shaped complexes; the angle between the two Fab arms varied between 10° for dimers to 180° in larger aggregates (159-161). These results seem to support a flexible model for IgG containing a hinge region. Such a flexible model was proposed by Noelken on the basis of hydrodynamic measurements (162). Optical rotatory dispersion measurements suggest that γ-globulin and Fab and Fc fragments have very little, if any, helical content (163,164).

Nature of the Antibody Combining Site

Many attempts have been made to determine the role played by the heavy and light chains in the antibody combining site. It is generally recognized that for full antibody activity both heavy and light chains must be present (165-169). However, the heavy chain alone appears to have the ability to bind antigen - albeit with a reduced affinity (170-172). Recently, the binding of N^{ε} DNP-L-lysine to light and heavy chains of DNP antibodies was studied by fluorescence quenching. It was found that the affinity of polyalanylated heavy chains for hapten was sixty times lower than that of intact antibodies, and no antibody activity was found to be associated with specific

light chains (173).

Other studies reveal a more complex picture. Pressman and Roholt (174), using the paired-labeling technique, implicated tyrosine residues in the light chain as being part of the antibody combining site. Affinity-labeling (176), with different reagents, of anti-hapten antibodies of various specificities showed that both the heavy and light chains were involved in the antibody site, although the ratio of the label in the heavy to light chains was 2:1 (177-180). In one study an affinity-labeled residue was identified as tyrosine-86 of the light chain (181).

Experiments involving the reconstitution of the antibody molecule from separated heavy and light chains have also been employed. When specific heavy chains and specific light chains were recombined, the activity of the heavy chains was enhanced. On the other hand, combination with non-specific light chains reduced, but did not abolish, the activity of the heavy chains (182). However, recombination of specific light with non-specific heavy chains produced a hybrid with negligible activity (172).

From these studies it may be suggested that the primary binding site resides in the heavy chain and that the role of the light chain is to stabilize the conformation of the active site on the heavy chain. This implicates the conformation of the antibody combining site as being an important factor in binding to antigen. It is not essential that the antibody combining site be composed of amino acid residues which are

sequential in the Fd fragment of the heavy chain. It is conceivable that the actual points of contact are separated from each other in the extended chain, but that by folding of the chain these are brought into close proximity to each other and, together, constitute a combining site. This view is supported by the fact that in the presence of hapten, Fab fragments are resistant to unfolding (183).

Studies with charged haptens have shown that antibodies directed against a positively charged group, e.g., phenyl-trimethylammonium, contain an extra negatively-charged aspartic acid residue and one more leucine residue on the heavy chain than does the heavy chain of anti-phenylarsonic acid antibody (184). Also, the combining sites of antibodies directed against non-polar haptens have been shown to be essentially hydrophobic in character (185,186). As was mentioned previously, upon N-octanoylation of a tripeptide of TMVP, the binding was increased, although the larger antigenic determinant consisted mainly of polar residues. This may indicate that the binding site of antibodies may possess some hydrophobic character regardless of the polar or apolar nature of the antigenic determinant. In this connection, the results of a study of the binding of various analogs of bradykinin to anti-bradykinin antibody are most interesting (187). This study revealed that changes in amino acid side-chains which altered charge or hydrophobic character had little effect on binding to antibody. However, change in length of the peptide chain was of greater importance. Moreover, alterations in residues

which force changes in conformation, e.g., glycine or proline, showed the most profound effect.

The size of the antibody combining site is relatively small, 25-34 x 12 x 7 Å, as indicated by inhibition studies discussed in a previous section (51). In an elegant study using a spin-labeled DNP hapten, electron spin resonance measurements showed that the depth of the combining site was 10 Å (188).

Heterogeneity of Antibodies

One of the most formidable problems facing the immunochemist is the heterogeneity of antibodies. Isotypic classes and subclasses of immunoglobulins are distinguishable by the structural properties of their heavy chains, γ, α, μ, δ, and ε (Table IV). In contrast, light chains fall primarily into two chemically different and antigenically distinct types, known as κ and λ. A normal human serum contains about 60% κ and about 30% λ chains, with smaller amounts of light chains possessing neither of the antigenic determinants of these two types. The κ and λ chains differ markedly in their amino acid composition and peptide maps (157).

Allotypic specificities, discovered by Oudin (189), are due to antigenic determinants of immunoglobulins which differ among normal individuals of the same species. The locale of these genetic markers differs from species to species. Idiotypic variations of immunoglobulins are found in different molecules of the same individual, and are localized on the Fab portion of the molecule (190,191).

In addition to the above-mentioned types of heterogeneity,

differences in antibody populations may be due to the nature of the immunizing antigen. As mentioned earlier, Sela found an inverse correlation between the net charge on the antigen and the antibodies formed. Using a variety of natural and synthetic antigens, the antibodies formed against the different molecules could be resolved into two chromatographically distinct fractions on DEAE-Sephadex (42,44). Antibodies formed against basic antigens were acidic and antibodies formed against acidic antigens were found to be more basic. Nor did the two fractions represent different subclasses of IgG. Moreover, immunization with the same haptenic group conjugated to basic and acidic carriers elicited both distinct types of antibodies even though each type possessed the same specificity (103,43). It was also indicated that the difference in charge between the two antibody fractions was not due to charge differences in the antibody combining site (192). These results suggest that in vivo recognition of the antigenic determinant occurs while the immunogen is still intact, i.e., prior to its degradation by proteolytic enzymes.

Differences in the antibody combining site result in heterogeneity of antibodies with respect to their affinity for the antigen. This has been confirmed in studies of the reversible binding of haptens by purified antibodies (8,193-196). Eisen and Siskind (196) showed that all populations of DNP antibodies, even those obtained from single bleedings of individual rabbits, were heterogeneous with respect to their affinity for hapten. Moreover, a progressive increase in

average association constants with increasing time of immunization was observed. These equilibrium constants, determined for the binding of N^{ϵ} DNP-lysine with different anti-DNP preparations, varied over a 10,000-fold range. Differences in amino acid composition of IgG antibodies of the same DNP specificity, obtained from rabbits that were homozygous at heavy and light chain loci, were observed (197). High affinity antibodies differed from low affinity ones in the same rabbit by having about four more tryptophan and about four more alanine residues.

Affinity labeling techniques have also revealed the heterogeneity of anti-hapten antibodies (85,185,198). Labeled anti-DNP light chains, obtained from single allotypically homozygous rabbit, were shown to be electrophoretically heterogeneous (179).

Attempts have been made to obtain a less heterogenous population of antibodies by immunizing with more structurally homogeneous antigens. Thus attachment of a single DNP group to papain (199) resulted in an antibody population which appeared to be electrophoretically less polydisperse. In contrast, immunization with mono-DNP-ribonuclease (200) and with DNP-insulin, in which the attachment site of the DNP group was regulated (201), resulted in as heterogeneous an antibody response as when DNP was coupled randomly to proteins. Immunization with pneumococcal (202) and streptococcal (203) polysaccharides has also resulted in the production of antibody populations of limited heterogeneity. In one study (204), p-aminobenzoate antibodies from a single rabbit were obtained

which were remarkably homogeneous and could be crystallized. However, this seems to be an unusual case since other studies using this antigen did not reveal this high degree of homogeneity.

From the foregoing discussion, it is evident that antibody heterogeneity constitutes a formidable problem for the immunochemist. The precise delineation of an antibody combining site must await more direct data such as can be provided by X-ray crystallography.

The discovery that some myeloma proteins have antibody-like activity and can combine specifically with the DNP group (205-207) offers much hope for the progress of the structural analysis of antibodies. These IgA myeloma proteins from human and mouse plasmacytomas, are monoclonal in origin and react homogeneously with the DNP group. Already, one myeloma protein has been crystallized and X-ray analysis has begun (208).

Methods of isolation of antibodies

The availability of pure antibodies is of prime importance for the investigation of the structure of antibodies and for the study of the physico-chemical mechanism underlying immunological reactions.

The procedures used for the isolation of antibodies may be divided into two groups: (a) non-specific methods which involve the fractionation of antisera on the basis of physico-chemical properties common to both antibody and non-antibody globulins, and (b) specific methods which take advantage of the specific combination of the antibodies with the appropriate antigen. Among the non-specific methods may be included precipitation

with appropriate salt concentrations, organic solvents and complexing agents, chromatography, electrophoresis and ultra-centrifugation. However, because of the closely similar physico-chemical properties of antibodies and normal globulins, these procedures usually result in preparations of antibody enriched by a factor of 10-20-fold rather than the isolation of purified antibodies.

The specific methods of isolation involve the following steps: (i) formation of insoluble antigen-antibody complexes, (ii) isolation of these complexes from other serum components, (iii) dissociation of these complexes under mild conditions, (iv) separation of the antibodies from the antigens. When the physical properties of antigens and antibodies are similar, this last step may involve considerable difficulty. This may be overcome by rendering the antigen insoluble during the dissociation process or by coupling it to insoluble and inert supporting media referred to as immunosorbents (209).

For the attachment of antigens to an insoluble backbone through covalent bonds, different natural and synthetic materials have been used such as cellulose (210), chemically modified cellulose (211-213), erythrocyte stroma (214), ion-exchange resins (215), polystyrene (216,217), copolymers of acrylamide, acrylic acid and methylene bisacrylamide (218), a copolymer of ethylene-maleic anhydride (EMA) (219), and more recently, Sephadex (220). In general hydrophilic polymers are more efficient than hydrophobic ones for reactions with proteins in aqueous solutions (221). Antibodies combine readily with the

homologous insolublized antigen and the complex can subsequently be dissociated at low pH. Moreover, since antibody-antigen complexes can be dissociated in excess hapten, solutions of appropriate haptens can be used for elution of antibodies from the immunosorbent, and the hapten can be subsequently removed by dialysis or gel filtration (222).

In general, the recovery of antibody is never 100% and the purity varies from 50-100% depending on the polymer used for the synthesis of the immunosorbent.

SCOPE OF THIS STUDY

The present investigation represents an attempt to further elucidate the chemical basis of antigenicity and the specificity of antibodies. Since the 3-dimensional structure and the antigenic regions of sperm-whale myoglobin have been well characterized, it was deemed advantageous to select the myoglobin-antimyoglobin system as a model for study.

In a previous investigation, the author synthesized one of the antigenic regions of myoglobin, viz., the C-terminal heptapeptide, and isolated the corresponding antibodies. That study demonstrated the general feasibility and usefulness of the synthetic approach in the study of protein antigen-antibody systems, and for the isolation of antibodies directed to well-defined regions of a natural protein antigen.

This thesis describes the synthesis of three of the lower homologs of the C-terminal heptapeptide, their use in the study of the more precise definition of the immunodominant region of the heptapeptide, and the isolation of the corresponding antibodies.

Attempts were made to study the binding between this region of myoglobin and the isolated antibodies by fluorescence quenching.

In addition, a second antigenic region, the tetradeca-peptide corresponding to a helical region of myoglobin (residues 56-69) was synthesized and its activity studied.

CHAPTER II

SOLID-PHASE SYNTHESIS OF PEPTIDE ANTIGENIC DETERMINANTS

General

The most important characteristics of life are the ability to synthesize biopolymers of defined sequence, and the transmission of this ability from one generation to the next. It is known that the information which is transmitted resides in the linear sequence of DNA, a class of heteropolymers composed of four monomeric units. This information is transcribed into another type of heteropolymer, RNA, and finally, in the cytoplasm, the information is translated into a third group of heteropolymers, the proteins, consisting of some twenty monomeric units, the amino acids. This last group is very important to the maint enance of life and, hence, it is extremely desirable to understand their sequences, mechanisms of synthesis and their modes of action. The peptide chemist attempts to answer such questions as, what is the relationship between chemical structure and biological activity; how does a hormone function and how can its action be regulated or modified; why are enzymes such specific and potent catalysts; what is the mechanism of the antigen-antibody reaction.

Historical

In 1901 the first peptide, glycyl-glycine, was synthesized by Emil Fischer using the acid chloride method (223). Subsequent introduction of the azide method of coupling by Curtius (224) led to molecules containing as many as nineteen

amino acids. This development was then followed by a latent period of about 20-25 years. In 1932 Bergmann and Zervas (225) revolutionized peptide chemistry with the introduction of the easily removable, amino protecting, carbobenzoxy group. This led to the synthesis of important peptides such as carnosine (226) and glutathione (227). The next breakthrough came in 1950 with the advent of the mixed anhydride method of coupling (228-231). This led, shortly afterwards to the Nobel Prize-winning synthesis of the pituitary hormone, oxytocin, in 1953 by du Vigneaud (232). This was the first definite proof that the information necessary for the biological activity of peptides and proteins lay in the amino acid sequence. The next ten years saw many improvements in protecting groups and coupling methods.

In 1962, R.B. Merrifield again revolutionized peptide chemistry by conceiving and exploring the radical idea of solid-phase synthesis (233-237). This method of synthesis, because of its speed and simplicity of operation has also been automated (238). This solid-phase method of synthesis will be discussed further in the next section.

To date, due to the advances in protecting groups and coupling methods, many naturally occuring peptide hormones and antibiotics have been synthesized. Recently, the list of polypeptides synthesized has been extended to include even proteins, e.g., insulin (239,240), ribonuclease (241,242), a protein containing 124 amino acids, and human growth hormone, which consists of 188 amino acid residues (243).

For a more comprehensive treatment of the tactics and strategies of peptide synthesis the reader is referred to review articles and monographs by Merrifield (244,245), Bodansky and Ondetti (246), Greenstein and Winitz (247), and Schroder and Lubke (248).

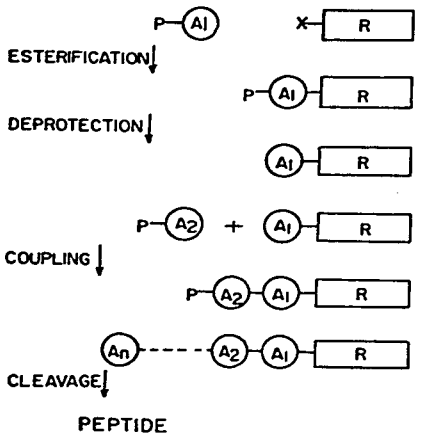
Solid-phase Peptide Synthesis

The basic principle of this method is that a peptide chain can be synthesized in a stepwise manner from one end while the other end is attached by means of a covalent bond to an insoluble, inert polymer. After the synthesis is complete, the entire peptide chain can be cleaved from the resin and isolated. This basic idea is outlined in Figure 4. The C-terminal amino acid of the proposed peptide chain (A_1) whose amino group is masked with a protecting group P, is attached by a covalent bond to the solid resin particle at a reactive site X. Group P can be selectively removed, exposing a new reactive amino group which is coupled with the carboxyl group of the second protected amino acid, A_2, to form the first peptide bond. The deprotection and coupling steps are repeated alternately until the required peptide is assembled. After completion of the synthesis the bond holding the peptide to the support is cleaved and the product, now free and in solution, is separated from the solid resin, generally by filtration, and purified. The greatest advantage of this method lies in the fact that the peptide, when bound to the insoluble support, is totally insoluble and hence can be separated from side-products and reagents, and efficiently washed by simple filtration.

Figure 4

Merrifield method of solid-phase peptide synthesis.

MERRIFIELD METHOD OF SOLID-PHASE PEPTIDE SYNTHESIS



X—R CHLOROMETHYLATED POLYSTYRENE WITH 2% DIVINYLBENZENE

A AMINO ACIDS

P PROTECTING GROUP

This affords a large saving of time and effort especially during the intermediate purification steps.

In general, the solid support used is a chloromethylated copolymer of 98% styrene and 2% divinylbenzene. The low cross-linking is sufficient to impart complete insolubility and good physical stability and still permit a high degree of swelling in organic solvents. The most commonly used amino protecting group is the t-butyloxycarbonyl group (BOC), while in some cases the carbobenzoxy group (Z) is used. However, the former is much more readily removed by anhydrous acids than is the latter.

Theoretically, almost any conventional coupling method can be used, but the two most frequently employed are the carbodiimide (249) and the active ester methods (250). In the coupling step it is most important that the reaction go to completion; otherwise peptides of intermediate length and lacking one or more amino acids would accumulate and contaminate the final product. (However, in practice 100% coupling is never quite achieved.) The preferred solvent for this step, for reactions mediated by carbodiimide, is methylene chloride since this solvent has a high dielectric constant, causes swelling of the resin and requires only a 1.5-fold molar excess (depending on the amino acid) of reagents as compared with a 3-fold molar excess in dimethylformamide.

EXPERIMENTAL

Materials

Chloromethylated polystyrene-2% divinylbenzene resin (containing 1.5 mequiv Cl/g) was purchased from Cyclo Chemical Co., Los Angeles, Calif. All protected amino acids were obtained from Schwartz BioResearch, Inc., Orangeburg, N.Y., and their purity was checked by thin-layer chromatography. Anhydrous hydrogen bromide and anhydrous hydrogen chloride were bought from Matheson, Coleman and Bell, East Rutherford, N.J. Concentrated hydrochloric acid, glacial acetic acid and aluminum oxide, chromatographic grade, were purchased from Baker Chemicals, Phillipsburg, N.J. Trifluoroacetic acid (TFA), dicyclohexylcarbodiimide (DCC) and triethylamine were obtained from Eastman Kodak Co., Rochester, N.Y. Absolute ethyl alcohol was supplied by Consolidated Alcohol, Toronto. Silica gel-H was purchased from Merck & Co., Rahway, N.J. DEAE-Sephadex was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents and solvents were obtained from Fisher Scientific Co., Montreal, and were of the highest grade commercially available.

Methods

Removal of Fines

The 'fines' were removed from the polystyrene resin by dispersing the swollen resin in methylene chloride in a separatory funnel. On standing, the resin rose to the top and the methylene chloride, containing the 'fines', was removed. This was repeated until the methylene chloride was clear. The resin was filtered

with suction, washed well with methanol and dried in air overnight.

Esterification

About 10 g of resin ('fines' removed) were swollen in 15 ml of ethanol in a 100-ml round bottom flask fitted with a reflux condenser. Moisture was excluded by fitting the condenser with a soda-lime tube. The BOC-amino acid* (7.5 mmole) was dissolved in 5 ml of ethanol and 0.19 ml (6.5 mmole) of triethylamine was added. The mixture was refluxed with stirring for 48 hours at 80-85° in an oil bath. The BOC-amino acid-resin was then filtered, washed well with ethanol and then with distilled water. Finally it was washed with methanol and dried overnight *in vacuo*.

Quantitative Estimation of Amino Acid Esterified to the Resin

About 50 mg of the BOC-amino acid-resin was stirred in 10 ml of 1 N HCl/acetic acid** for 30 minutes. It was then collected on a filter, washed with glacial acetic acid, distilled water and finally with methanol. The material was dried overnight *in vacuo*.

The amino acid was then liberated from the resin by hydrolysis. Forty mg of amino acid-resin was refluxed in dioxane***/HCl, 1:1, in a 100-ml round bottom flask fitted with a long air

---

*All amino acids used in this study were of the L-configuration.

**Prepared by bubbling anhydrous HCl into 200 ml of glacial acetic acid for 45 minutes. The normality of the HCl was determined by Volhard analysis, and was adjusted by diluting with acetic acid (253).

***Dioxane was rendered peroxide-free by passing through an activated alumina column and testing with KI.

condenser for 18 hours. The material was filtered, the resin washed with 0.1 N HCl and the washings added to the filtrate which was evaporated to dryness under reduced pressure. Water was added to the residue and evaporated to dryness. This was repeated three times. The sample was then analyzed for amino acid content.

Peptide Formation

The procedure followed for the synthesis of the peptides was that outlined by Merrifield (235) with minor modifications (254). BOC-amino acids were used except when lysine occurred as the N-terminal residue in which case bis-Z-lysine was coupled. BOC-amino acids with protected side chains were O-benzyl-tyrosine, γ-benzyl-glutamic acid, β-benzyl-aspartic acid, im-benzyl-histidine, O-benzyl-serine, O-benzyl-threonine, and N^{ε} -Z-lysine. All coupling reactions were mediated with DCC* except that involving the carboxyl group of glutamine which was coupled directly as the p-nitrophenyl ester.

In a typical synthesis, the dried BOC-amino acid-resin was introduced into the reaction vessel**, suspended in 30 ml of methylene chloride and rocked for about 30 minutes in order to swell the resin. All subsequent reactions were performed excluding moisture. The cycle of reactions that was followed

---

*This reagent produces severe skin irritation. Gloves and goggles should be worn when handling.

**The reaction vessel consisted of a cylindrical glass vessel (13 x 4 cm) with a coarse sintered-glass disk at one end. About 9 cm from the disk was an opening (fitted with a soda-lime tube) through which reagents were introduced into the vessel.

to introduce each new residue is outlined in Table V.

After completion of the cycle introducing the last amino acid to be incorporated the peptide-resin was removed from the vessel, filtered, washed well with methylene chloride, ethanol and methanol. The material was dried over P_2O_5 *in vacuo*.

Synthesis of the C-terminal Heptapeptide and Homologs

The C-terminal heptapeptide of sperm-whale myoglobin, Lys-Glu-Leu-Gly-Tyr-Gln-Gly, and its homologs, Glu-Leu-Gly-Tyr-Gln-Gly, Leu-Gly-Tyr-Gln-Gly, and Gly-Tyr-Gln-Gly were synthesized as follows: BOC-glycine-resin (7 g containing 0.20 mmole/g resin) was introduced into the synthesis vessel and subjected to three cycles of deprotection, neutralization and coupling. After coupling the fourth residue (glycine), a portion of the tetradecapeptide-resin was removed from the reaction vessel. This was repeated after the fifth and sixth residues were coupled. Each protected peptide-resin was stored *in vacuo* over P_2O_5 prior to subsequent cleavage.

Synthesis of the Tetradecapeptide (Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly-Val-Thr-Val-Leu)

For the synthesis of the protected tetradecapeptide, 5 g of BOC-leucine-resin (0.25 mmole/g resin) was used at the start. Thirteen cycles of deprotection, neutralization and coupling were carried out. At the conclusion of the synthesis the protected peptide-resin was removed from the vessel, collected on a filter, washed with methylene chloride, ethanol and methanol, and dried *in vacuo* over P_2O_5.

After cleavage from the resin (*vide infra*), the *im*-benzyl protecting group was removed by reduction with sodium in liquid

TABLE V

One cycle of solid-phase peptide synthesis.

TABLE V

Step	Reagent	Purpose	No. of Applications	Volume (ml)	Time (min) per Application
1	HOAc*	washing	3	40	3
2	1 N HCl/HOAc**(TFA)	deprotection	2	40	15
3	HOAc	washing	3	40	3
4	EtOH	washing	3	40	3
5	$CHCl_3$	washing	3	40	3
6	$Et_3N/CHCl_3$ (1:30)	neutralization	1	30	10
7	$CHCl_3$	washing	3	40	3
8	CH_2Cl_2***	washing	3	40	3
9	BOC-amino acid in CH_2Cl_2+	mixing	1	25	5
10	DCC++	coupling	1		5 hours
11	CH_2Cl_2	washing	3	40	3
12	EtOH	washing	3	40	3

*Abbreviations used are: HOAc, glacial acetic acid; EtOH, ethanol; Et_3N, triethylamine; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid.

**To deprotect BOC-glutamine TFA was used.

***When BOC-glutamine-p-nitrophenylester was introduced dimethylformamide was used in steps 8, 9 and 11.

+A 3-fold molar excess of each amino acid derivative was used.

++A stoichiometric amount of DCC was added in the form of a 50% solution in CH_2Cl_2. This step was omitted when BOC-glutamine-p-nitrophenylester was coupled.

ammonia (226) as follows: about 300 ml of liquid ammonia was dried over sodium. It was then redistilled under anhydrous conditions and collected in a 3-necked round bottom flask at -70°. The im-benzyl-tetradecapeptide (150 mg) was then dissolved in the dry, distilled ammonia solution with vigorous stirring. The peptide-ammonia solution, upon boiling (-30°), was titrated with sodium* with the exclusion of moisture, until a permanent light blue color remained. After a few seconds the reaction was quenched by adding a few drops of dry glacial acetic acid (255). The ammonia was removed by connecting the flask to a water aspirator with two soda-lime drying tubes in the line to avoid moisture. When the flask was dry, the crude deprotected peptide was dissolved in acetic acid and freeze-dried.

Synthesis of N^{α} DNP, N^{ε} Z-lysine

N^{α} BOC, N^{ε} Z-lysine (1.4 g) was stirred in TFA for 30 minutes to remove the BOC protecting group. The TFA was evaporated off on a rotary evaporator under reduced pressure. The residue was neutralized with 1 M $NaHCO_3$ and the resulting precipitate was collected on a filter, washed well with water and dried over P_2O_5 *in vacuo*.

Dry N^{ε} Z-lysine (700 mg) was suspended in 15 ml of a mixture of 0.1 N $NaHCO_3$/methanol, 8:3, and 0.9 ml of 1-fluoro, 2,4-dinitrobenzene in 15 ml of methanol was added slowly with

*This was accomplished with the use of a 'sodium stick'. Liquified sodium was drawn into a 1 ml pipet and fitted via a rubber stopper into the reaction vessel. The pipet was greased for easy movement. The titration was performed by a series of very quick immersions of the tip of the sodium stick into the ammonia solution.

stirring. The reaction was allowed to proceed overnight in the dark. N^{α} DNP, N^{ϵ} Z-lysine is light-sensitive, consequently all subsequent operations were performed as much as possible with the exclusion of light.

The bright yellow-orange suspension was extracted with 3-50 ml portions of ether and then acidified with 50% citric acid to pH 2. The cold, acidified suspension was treated with 3-75 ml portions of cold ethyl acetate. The combined cold ethyl acetate extract was then washed with cold water and dried over $MgSO_4$. It was then filtered, and the filtrate was evaporated on a rotary evaporator under reduced pressure.

The oily residue was analyzed by thin-layer chromatography on silica gel-H in n-butanol/acetic acid/water, 4:1:1, and also in chloroform/methanol/acetic acid, 98:2:1. The dried plates were sprayed with ninhydrin. Bis-DNP-lysine and N^{ϵ} Z-lysine were run as controls.

Synthesis of N^{α} DNP-Lys-Glu-Leu-Gly-Tyr-Gln-Gly

The N^{α} DNP, N^{ϵ} Z-lysine was coupled to 1.62 g of Glu-Leu-Gly-Tyr-Gln-Gly-resin, and allowed to react overnight in the dark. After completion of the cycle, the dark-brown, protected N^{α} DNP-heptapeptide-resin was removed from the vessel, collected on a filter, washed with methylene chloride, ethanol and methanol, and dried over P_2O_5 *in vacuo*. The material was stored in the dark.

Cleavage of the Peptides from the Resin

The following general procedure was followed to remove the individual peptides from each peptide-resin. The protected

peptide-resin was suspended in a mixture of TFA/methylene chloride, 1:1, containing 50 mmole of anisole for each mmole of tyrosine (256). A slow stream of anhydrous HBr was bubbled into the solution for 70 minutes with the exclusion of moisture. The HBr was first scrubbed by passing it through a solution of resorcinol in TFA. The free peptide was filtered and the resin washed several times with TFA. The combined filtrates were evaporated at 10° on a rotary evaporator under reduced pressure.

The oily residue was triturated with ether and the precipitated peptide filtered and redissolved in a small volume of TFA. It was reprecipitated with ether and filtered. The peptide was then dissolved in a suitable solvent such as water, in the case of the C-terminal peptides, or acetic acid, in the case of the tetradecapeptide, and freeze-dried.

Purification of the Peptides

The C-terminal peptides, including the DNP-heptapeptide, were purified by chromatography on a 1.1 x 40 cm column of DEAE-Sephadex, A-25, equilibrated with 0.01 M NH_4HCO_3. Elution was accomplished with a gradient of 0.01 M - 0.15 M NH_4HCO_3. To form the gradient, a nine-chambered Buchler Varigrad was used. The 0.01 M NH_4HCO_3 (160 ml) was introduced into the first chamber and 160 ml of the 0.15 M NH_4HCO_3 was introduced into each of the remaining eight chambers. The effluent was continuously monitored at 220 nm by a Gilford Model 2000 multiple sample absorbance recorder equipped with a Beckman DU monochromator. In this way the solvent absorption was continuously corrected for.

All operations involving the DNP-heptapeptide were performed with the exclusion of light.

Thin-layer Chromatography

Silica gel-H (35 g) was homogeneously dispersed in 70 ml of glass-distilled water. The suspension was layered evenly on glass plates (20 x 20 cm) and allowed to dry at room temperature overnight.

The solvent systems used for analysis of the peptides were n-butanol/acetic acid/water, 4:1:5, (upper phase) as well as 75% phenol. Solvent systems used for the analysis of N^{α} DNP, N^{ε} Z-lysine were n-butanol/acetic acid/water, 4:1:1, and chloroform/methanol/acetic acid, 98:2:1.

The chromatography tank was filled with 100 ml of the appropriate solvent system and the sides of the tank lined with filter paper soaked in the solvent. The closed tank was equilibrated in this way for several hours.

About 1 mg of sample was dissolved in 0.1 ml of the appropriate solvent and about 10-20 µl applied to the plate by means of a capillary. The chromatograms were developed for 60-90 minutes. The plate was then removed from the tank and the solvent front marked as quickly as possible. It was thoroughly dried and sprayed with ninhydrin. The tetradecapeptide was sprayed first with Pauly reagent, then with ninhydrin.

Amino Acid Analysis

Samples of the products to be analyzed were hydrolyzed in 6 N HCl *in vacuo* at 110° for 20 hours. Analyses were performed on a Beckman Amino Acid Analyzer, Model 120B, according to Spackman *et al.* (257).

RESULTS

Purification of the C-terminal Peptides

The elution patterns of the C-terminal peptides chromatographed on DEAE-Sephadex are shown in Figure 5. As can be seen, as each residue is added to the growing peptide chain the complexity of the pattern obtained is increased, indicating the importance of complete coupling at each step. In this study no effort was made to monitor the degree of completion of each coupling reaction. In each elution pattern, at least one peak represents material which is deficient in tyrosine, as indicated by its failure to absorb at 280 nm.

The results of the amino acid analyses and thin-layer chromatography of the C-terminal peptides are summarized in Table VI. The C-terminal peptides each revealed only one spot by thin-layer chromatography, which, when coupled with the amino acid analyses, revealed a high degree of purity. The lower molar ratios for tyrosine obtained by amino acid analysis can be explained by the fact that this residue is somewhat acid sensitive and is partially destroyed under the conditions of hydrolysis employed here.

The identity of the DNP-heptapeptide was established by its simultaneous absorption at 220, 280 and 360 nm, and it was not analyzed by amino acid analysis.

Thin-layer chromatography of N^{α} DNP, N^{ε} Z-lysine in n-butanol/acetic acid/water, 4:1:1, revealed only a single spot (R_f 0.70), while chromatography in chloroform/methanol/acetic acid, 98:2:1, showed one major spot (R_f 0.29) and a slight trace

Figure 5

Elution patterns of the C-terminal peptides. A column (1.1 x 40 cm) of DEAE-Sephadex was equilibrated with 0.01 M NH_4HCO_3. Elution was accomplished with a gradient from 0.01 M to 0.15 M NH_4HCO_3 (arrow indicates start of gradient). (a) N^{α} DNP-heptapeptide; (b) heptapeptide; (c) hexapeptide; (d) pentapeptide; (e) tetrapeptide.

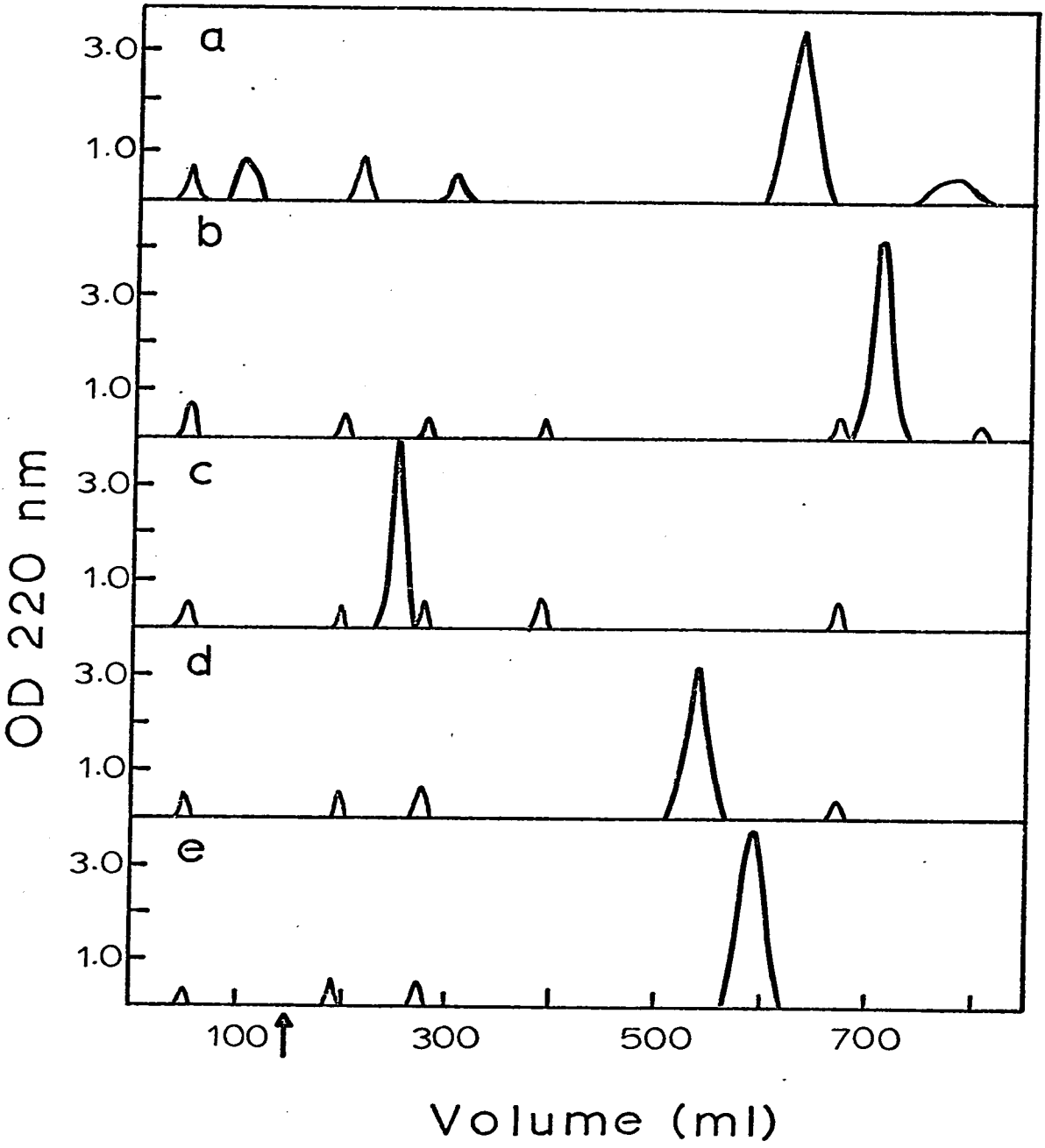

a
b
c
d
e
3.0
1.0
OD 220 nm
100
300
500
700
Volume (ml)

TABLE VI

Analysis of Peptides.

TABLE VI

Peptide	Sequence	Molar Ratio of Amino Acids					R_f	
		Gly	Glu	Tyr	Leu	Lys	A*	B
Tetrapeptide	Gly-Tyr-Gln-Gly	2.00	1.00	0.80			0.15	0.28
Pentapeptide	Leu-Gly-Tyr-Gln-Gly	2.00	0.99	0.91	1.02		0.23	0.44
Hexapeptide	Glu-Leu-Gly-Tyr-Gln-Gly	2.00	2.09	0.86	1.01		0.14	0.27
Heptapeptide	Lys-Glu-Leu-Gly-Tyr-Gln-Gly	2.00	2.00	0.86	1.00	1.09	0.13	0.23
DNP-heptapeptide	N^{α} DNP-Lys-Glu-Leu-Gly-Tyr-Gln-Gly						0.22	0.39

*Solvent system A, n-butanol/acetic acid/water, 4:1:5; System B, 75% phenol.

of dinitrophenol. Spraying with ninhydrin did not reveal any additional spots indicating that there were no free amino groups present. Bis-DNP-lysine and N^{ϵ} Z-lysine were run as controls and did not correspond to any material. This was true in both solvent systems.

Synthesis and Analysis of the Tetradecapeptide

Upon completion of the synthesis of the protected tetradecapeptide, the weight increase of the resin was 1.52 g. After cleavage of the peptide from the resin with HBr, the weight of im-benzyl-tetradecapeptide was 1.34 g which represents a yield of 60% based on the amount of leucine esterified to the resin at the beginning of the synthesis. Reduction with sodium in liquid ammonia reduced the yield of free tetradecapeptide to 43% overall.

Thin-layer chromatography of the crude tetradecapeptide revealed one major spot and four minor contaminants with both solvent systems. The plate chromatographed in n-butanol/acetic acid/water, 4:1:5, was sprayed first with Pauly reagent and subsequent spraying with ninhydrin did not change the pattern. No spot could be detected corresponding to im-benzyl-tetradecapeptide indicating that the im-benzyl group was completely removed. The R_f value of the major spot chromatographed in 75% phenol was 0.42. The tetradecapeptide was used without further purification to elute antibody from the myoglobin immunosorbent (see next chapter).

DISCUSSION

The success of the solid-phase method of peptide synthesis is critically dependent on the completion of each coupling reaction. Unless each coupling reaction goes to 100% completion failure sequences can occur (258), in which one or more amino acids are missing from within the chain. That this is indeed the case seems evident from the heterogeneous patterns obtained from the purification of the peptides on DEAE-Sephadex. Although it is also possible that at least some of the complexity of the elution profiles could be due to partial destruction during the cleavage step. However, it has been found that peptide bonds involving amino acids with bulky side chains are not formed as readily as others (259). This could explain the fact that at least one component of each synthesis of the C-terminal peptides, upon separation on DEAE-Sephadex, did not contain tyrosine as evidenced by the lack of absorption at 280 nm. No attempt was made in this study to monitor the degree of coupling at each step. However, in order to minimize this possibility of failure sequences, large excesses of activated amino acids were introduced. In these syntheses a 3-fold molar excess of each amino acid was used.

In addition to failure sequences, truncated peptides may also occur, in which the chain growth is terminated before the desired length is obtained. One of the difficulties in the syntheses of the C-terminal peptides is the possibility of cyclization of the glutamine residue during and after the deprotection step. This, of course, would arrest the growth

of the peptide chain. To diminish this possibility, a milder cleavage of the BOC group of glutamine was used, i.e., cleavage with TFA instead of with 1 N HCl/acetic acid (260,261).

To incorporate glutamine into the peptide chain the use of the p-nitrophenyl ester derivative is preferable since the use of the diimide reagent can result in the formation of the nitrile derivative (262,263). With the p-nitrophenyl ester, the preferred solvent was dimethylformamide, since in this solvent the condensation proceeds quite rapidly.

In the sequential synthesis of the C-terminal peptides no effort was made to determine percent yields since unknown amounts of the peptide-resin were removed each time. Using this method, estimation of percent yields of peptides is almost impossible. However, in cases where the heptapeptide was synthesized directly, without removal of material prior to incorporation of the final residue, the yield of crude protected peptide, as measured by weight increase of the resin, was about 80-90%. However, after cleavage and purification, the yield of purified product was usually about 50% based on the amount of glycine esterified to the resin at the start of the synthesis.

In the synthesis of the tetradecapeptide, the introduction of several bulky amino acid derivatives, e.g., BOC-im-benzyl-histidine, BOC-O-benzyl-threonine and BOC-O-benzyl-serine, probably resulted in some failure sequences also. In addition, removal of the im-benzyl group by reduction with sodium in liquid ammonia, a rather harsh treatment, may have resulted in some fragmentation of the peptide chain. To circumvent the

use of reduction with sodium in liquid ammonia, the imidazole side chain may be protected with a DNP group (264) which is easily removable under milder conditions (265).

The yield of im-benzyl-tetradecapeptide was only 60% of the theoretical yield. As can be seen, with increasing chain length the efficiency of the synthesis decreases considerably.

The general strategy employed by the Merrifield method of solid-phase peptide synthesis is that of stepwise elongation of the peptide chain starting from the carboxyl end. The advantage of this strategy is the general freedom from racemization. In this technique, the carboxyl groups of single amino acids are activated, as opposed to the carboxyl groups of peptides, which would be the case in starting from the amino end. It has been found that this approach assures freedom from racemization, not only in solid-phase synthesis but also in classical methods of peptide synthesis in solution. This is especially true when urethane groups, e.g., carbobenzoxy, or t-butyloxycarbonyl, are used to protect the amino function. It need not be stressed that racemization of peptides to be used for immunochemical studies would pose a serious problem.

In general, the Merrifield method of solid-phase peptide synthesis has proved to be quite successful in the synthesis of the peptides related to two immunologically active regions of myoglobin. The yields were superior to those that could be obtained by classical methods of synthesis. In addition, the time required for synthesis by the solid-phase technique is drastically reduced.

CHAPTER III

IMMUNOCHEMICAL STUDIES WITH SYNTHETIC PEPTIDES

Introduction

This chapter describes the use of the synthetically prepared peptides in an attempt to establish a more precise delineation of the immunodominant region of the antigenic site corresponding to the C-terminal heptapeptide of myoglobin as well as the antigenic activity of the tetradecapeptide corresponding to the helical region in myoglobin comprising residues 56-69 (73). An effort was also made to measure, by fluorescence quenching, the binding between the purified antibodies to the C-terminal heptapeptide and (i) the N^{α} DNP derivative of the heptapeptide, and (ii) the intact myoglobin molecule.

Fluorescence Quenching

The technique of fluorescence quenching has recently found much application in the study of binding between haptens and antibodies (196,266,267), as well as between haptens and antibody sub-units (173).

Proteins containing tyrosine and tryptophan residues, when excited with ultraviolet light at a wavelength corresponding to their absorption maxima, emit light in the region of 330-350 nm, and are said to fluoresce. Antibodies, since they are proteins containing these residues, also exhibit fluorescence. However, the fluorescence spectrum of antibodies corresponds with that of tryptophan and not of tyrosine (266). When certain ligands bind antibody molecules the radiationless transfer of excitation

energy from the antibody to the bound ligand results in a damping or quenching of the antibody's fluorescence. However, the efficiency of this energy transfer depends on the overlap of the absorption spectrum of the ligand with the fluorescence spectrum of the antibodies, i.e., when the bound ligand absorbs in the region 330-360 nm (268). In addition, for efficient transfer of excitation energy to occur, certain geometric requirements must be met, i.e., the geometric orientation of the bound ligand with respect to the tryptophan residues located at or near the antibody combining site plays an important role in quenching (269).

Using this technique, the reversible binding of antibodies and haptens may be measured quantitatively, provided, of course, the above conditions are met. Although fluorescence quenching is an indirect, empirical method it has the advantage over more direct methods, e.g., equilibrium dialysis, in that only very small amounts of antibody are needed, and the measurements are made readily within only a few minutes. The rapidity of this technique makes it possible for binding measurements to be made under extreme conditions of pH and temperature. Moreover, binding between univalent antibody fragments and large antigens may also be measured by this technique, since these complexes are soluble. However, the method is limited by the fact that purified antibody preparations must be used since the fluorescence of other proteins present in serum would tend to obscure the quenching due to the specific interaction of antibodies and haptens. In addition, since many haptens may

not produce quenching, this technique is less generally applicable than equilibrium dialysis.

Much of the work done in this area has been done using the DNP and azobenzene systems since these systems fulfill the spectral conditions necessary for energy transfer (173,196, 266,267,270,271).

For a more comprehensive treatment of the theory and fundamental nature of the processes involved in fluorescence and fluorescence quenching, the reader is referred to articles and monographs by Velick (269), Hercules (268), Konev (272), and Seliger and McElroy (273).

EXPERIMENTAL

Materials

Sperm-whale skeletal muscle was generously donated by Western Canada Whaling Co. Ltd., Vancouver, B.C. Cellulose was purchased from Whatman, England. Complete Freund's Adjuvant and Noble Agar were obtained from Difco Laboratories, Detroit, Michigan. Sephadex G-10 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Normal rabbit γ-globulin (RGG) was purchased from Pentex Inc., Kankakee, Illinois. 'No Screen' X-ray film was bought from Kodak Film Co. ^{125}I was supplied by Charles E. Frosst and Co., Montreal. All chemicals and reagents used in this investigation were reagent grade unless otherwise specified.

Methods

Isolation of Myoglobin

Approximately 5 lb of sperm-whale meat was thawed at4°C and minced. Myoglobin was subsequently extracted and isolated by the zinc-ethanol procedure of Hardman et al (274). It was then purified by $(NH_4)_2SO_4$ precipitation at 4°C. To a 600 ml solution of myoglobin, solid $(NH_4)_2SO_4$ was added to 50% saturation. The pH was maintained between 6.5 and 7.0 by addition of 0.5 N NaOH. At 50% saturation the solution was filtered by suction and the precipitate, mainly non-heme proteins, was discarded. The $(NH_4)_2SO_4$ concentration was raised by 3% increments, and aliquots of the suspension filtered. Each time the solution was filtered, samples of the filtrate and of the precipitate were each diluted with water and the spectra recorded.

The approximate percentage of myoglobin present was determined by comparing absorbances at the 418 nm peak (the Soret peak of oxymyoglobin) and the 280 nm peak. The molar extinction coefficients of oxymyoglobin at these two wavelengths are 12.8×10^4 and 3.16×10^4 respectively (274).

When the filtrate was about 90-95% myoglobin (in some cases it was 97%) the $(NH_4)_2SO_4$ concentration was raised to 80% saturation and the myoglobin was collected by centrifugation, washed with saturated $(NH_4)_2SO_4$ solution and recentrifuged. The crystalline precipitate was then resuspended in a small volume of saturated $(NH_4)_2SO_4$ and stored as a paste at 4°. Myoglobin thus prepared, was further purified* on IRC-50 by the procedure of Edmundson (73).

Preparation of Antisera

Antisera to myoglobin were obtained by intradermal injections, at multiple sites, into the lower abdominal region of albino rabbits. A total of 1 ml of solution, consisting of 0.5 ml of 2% myoglobin dissolved in saline**, mixed with 0.5 ml of complete Freund's adjuvant, was used for each injection. The first series of injections was followed two weeks later by a second series of injections totalling 1 ml and consisting of 0.5 ml of 4% myoglobin solution mixed with 0.5 ml of adjuvant. Following this, the animals were injected intradermally at monthly intervals

---

*The author is indebted to Dr. D. Ajdukovic for this final purification.

**The term saline is used throughout this thesis to denote a 0.9% solution of NaCl.

with a solution consisting of 1 ml of equal volumes of 4% myoglobin and adjuvant. The animals were bled ten days after each injection. All sera were stored at -20° and were used individually.

Preparation of Immunosorbents

Cellulose was bromoacetylated according to Robbins et al. (213). A sample of wet paste was dried in vacuo over P_2O_5 and the dry weight was determined. The wet paste consisted of 13-15% bromoacetylcellulose.

(i) Bromoacetylcellulose-Heptapeptide Immunosorbent (BAC-H)

Bromoacetylcellulose (0.3 g dry weight) was suspended in 6 ml of 0.15 M phosphate-citrate buffer, pH 4.0 and 10 mg of heptapeptide, dissolved in 1 ml buffer was added. The suspension was stirred overnight at room temperature, centrifuged and resuspended in 8 ml of 0.1 M $NaHCO_3$, pH 8.9. It was then allowed to stand at 4° for 24 hours with occasional gentle stirring. After centrifugation, it was resuspended in 10 ml of 0.1 M $NaHCO_3$ containing a few drops of ethanolamine, pH 9, and allowed to stand overnight at 4°. The suspension was then centrifuged, washed several times with PBS*, resuspended in 8 M urea and allowed to stand overnight at 4°. It was then washed well with PBS. To simulate the conditions for elution, it was incubated at 37° for 1 hour in 0.15 M glycine-HCl buffer, pH 2.5. The immunosorbent was centrifuged, washed well with PBS and stored at 4° until ready for use.

---

*PBS is used throughout this thesis to denote a solution of phosphate-buffered saline, pH 7.1, unless otherwise stated.

(ii) Bromoacetylcellulose-Myoglobin Immunosorbent (BAC-Mb)

Myoglobin (100 mg) was adsorbed to 0.5 g of bromoacetyl-cellulose in 0.1 M acetate buffer, pH 4.0. It was coupled and washed as described above.

Isolation of Antibodies

The antibodies to the C-terminal heptapeptide, and to the tetradecapeptide were isolated from BAC-H and/or BAC-Mb using a batchwise technique. The general strategies of the methods are outlined diagramatically in Figures 6 and 7.

Rabbit anti-myoglobin sera were incubated with the immunosorbents for 1 hour at 37° and overnight at 4°. The suspension was centrifuged at 20,000 g and washed with PBS until the $O.D._{280}$ was 0.01. The immunosorbents were then treated with 10 μmole/ml of peptide, and/or 0.15 M glycine-HCl, pH 2.5 and 2.0. In some cases the immunosorbents were eluted directly with glycine-HCl, pH 2.5 and pH 2.0. Each eluant solution was incubated with the immunosorbent for 1 hour at 37°. The suspension was centrifuged at 20,000 g and the supernatant eluate passed through a millipore filter (0.45 μ porosity). After incubation with eluant solution the immunosorbent was washed thoroughly with PBS (in the case of the peptides) or with glycine-HCl, until the $O.D._{280}$ was 0.01.

The purified antibody solutions were dialyzed against 0.1 M NH_4HCO_3 when eluted with peptide, followed by exhaustive dialysis against PBS. Otherwise, the eluates were dialyzed directly against PBS. The O.D. at 280 nm of the eluates was then recorded. The eluates were concentrated by negative pressure dialysis.

Figure 6

Schematic strategy for the isolation of antibodies to a single antigenic determinant using insolubilized myoglobin. (1) BAC-Mb immunosorbent. The various antigenic determinants of myoglobin are represented. (2) Absorption of the immunosorbent with anti-myoglobin serum. The antibodies of various specificities associate with their respective determinants. (3) Elution with excess synthetic peptide. Displacement of the equilibrium in favor of dissociation takes place and the antibodies directed to the single antigenic determinant can be eluted and isolated.

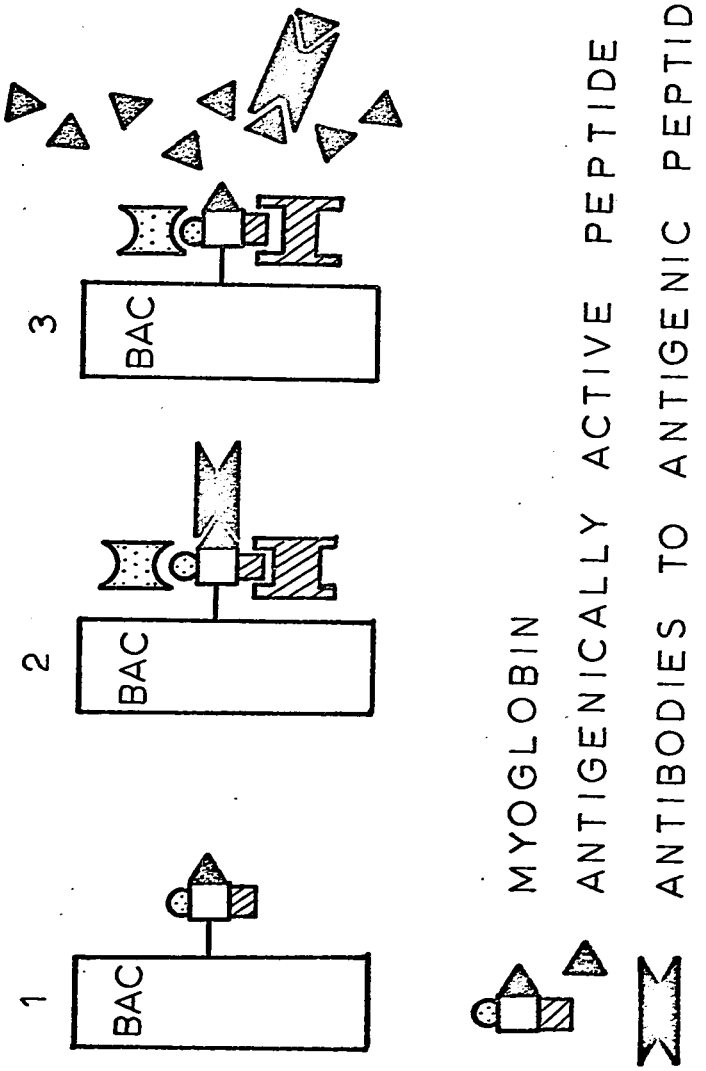
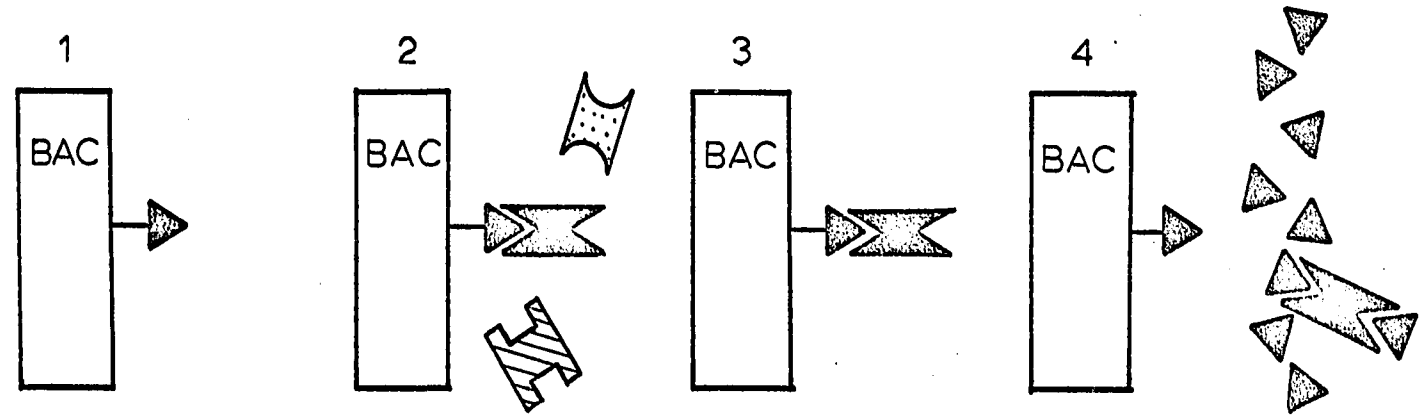

1
BAC
2
BAC
3
BAC
MYOGLOBIN
ANTIGENICALLY ACTIVE PEPTIDE
ANTIBODIES TO ANTIGENIC PEPTIDE

Figure 7

Schematic strategy for the isolation of antibodies to a single antigenic determinant using insolubilized antigenically active peptide. (1) BAC-peptide immunosorbent containing only one antigenic determinant. (2) Absorption of the immunosorbent with anti-myoglobin serum. Only antibodies directed against the one antigenic determinant are absorbed. (3) Removal of antibodies of other specificities by washing. (4) Elution with excess peptide. Displacement of the equilibrium in favor of dissociation takes place, and the antibodies directed to the single antigenic determinant can be eluted and isolated.



ANTIGENICALLY ACTIVE PEPTIDE

ANTIBODIES TO ANTIGENIC PEPTIDE

ANTIBODIES TO OTHER ANTIGENIC DETERMINANTS

The peptide could be recovered from the NH_4HCO_3 dialysate by evaporating off the solvent and bulk of the salt *in vacuo* at 40° followed by gel filtration on Sephadex G-10.

Disc Electrophoresis

The eluted antibodies were analyzed by electrophoresis in 7% polyacrylamide gel using Tris-glycine buffer, pH 8.3, as described by Ornstein and Davis (275), using the standard apparatus obtained from Buchler, Fort Lee, N.J. Instead of using the 'stacking gel' the density of the samples was increased with sucrose. Runs were performed at 5 mA per tube for 45 minutes. The gels were subsequently stained overnight with amidoblack, and destained electrolytically at 6 mA per tube.

The gels were stored in stoppered tubes in 7% acetic acid.

Immunodiffusion

Gel diffusion in two dimensions was performed according to the procedure of Ouchterlony (19) with some modifications. Solutions of 1.2% Noble agar in saline were poured into 5 cm diameter disposable Petri dishes (5 ml per dish). The holes were formed by placing 7 brass cylinders (5 mm diameter) in appropriate positions in the dishes prior to adding the agar. One cylinder was placed in the center of the dish while the other six formed a circle around it. The agar was allowed to gel at room temperature.

The center well was filled with 0.05% myoglobin solution in saline and the outer wells were filled with the antibody solutions to be tested for activity. Normal rabbit serum (NRS)

as well as the original antimyoglobin serum were placed in separate outer wells and run as controls. Diffusion was allowed to take place for 24 hours at room temperature, but the plates were inspected every 2 hours after 16 hours had elapsed.

Radioiodination of Myoglobin

Myoglobin was radioiodinated with ^{125}I according to the procedure of Yagi et al. (216) with minor modifications. To 3 mc of ^{125}I was added 0.1 ml of KI (8×10^{-4} in PBS, pH 7.5), 0.2 ml Chloramine-T (0.1 M), and 2 mg of myoglobin dissolved in 0.2 ml PBS, pH 7.5. The mixture was allowed to react for 5-6 minutes at room temperature when 0.1 ml of 0.3 M $NaSO_3$ was added. Free ^{125}I was removed from iodinated myoglobin by passing the reaction mixture through a Dowex-1 x 8 column (1 x 15 cm) equilibrated with PBS, pH 6.8. The activity of the myoglobin was about 10^8 cpm/mg. Radioactivity measurements were made with a Nuclear Chicago Gamma-Ray Counter, the emission band with energy at 35.5 KeV being measured.

Radioimmunodiffusion

Radioimmunodiffusion was performed in 1.2% Noble agar in PBS containing 0.5% sodium azide to prevent bacterial growth. The slides were 'painted' with agar and dried. Three ml of molten agar was poured on the slides and allowed to gel. Wells of 3 mm diameter were made with an LKB hole puncher*(see Figure 10). A volume of 10 µl was applied to each well.

The eluted antibodies were mixed with normal RGG, and were

---

*LKB-Produkter BA, Stockholm, Sweden.

allowed to diffuse against sheep anti-rabbit serum for 24 hours at room temperature in a humid chamber. RGG, alone, was also diffused against the sheep antiserum as a control. After diffusion, the excess sheep antiserum was removed by washing the slides in PBS for several hours. Radioiodinated myoglobin was then applied to the same holes as the sheep antiserum and allowed to diffuse for 24 hours at room temperature. The slides were then washed exhaustively in PBS (containing 0.5% sodium azide) until the radioactivity of the washings approached that of background level. They were then washed in distilled water.

The slides were thoroughly dried and wrapped in a thin sheet of saran (Dow Chemical Co., Midland, Michigan). Kodak 'No Screen' X-Ray film* was fixed over the slides for three days in a light-tight container. They were then stained with carbofuscein dye.

The films were developed in Kodak Liquid X-Ray Developer and Replenisher and fixed in Kodak Liquid X-Ray Fixer. Both processes were performed according to the manufacturer's instructions.

Ultracentrifugation

Ultracentrifugal analysis was performed in a Spinco Model E ultracentrifuge. A synthetic boundary cell was used and the run performed at 20° with a speed of 59,780 rpm. The solvent was PBS. Photographs were taken every four minutes after

*The X-ray film was always handled in total darkness.

attaining full speed.

Concentration Measurements

Protein concentrations were measured in a Zeiss PMQ II spectrophotometer. The optical density of solutions of γ-globulin, myoglobin and DNP-heptapeptide were measured at 280, 409 and 360 nm, respectively. Concentrations were calculated using a molar extinction coefficient of 22.5×10^4 and assuming a molecular weight of 150,000 for γ-globulin. Molar extinction coefficients used for myoglobin (274) and N^{α} DNP-heptapeptide were 16.6×10^4 and 17,000* respectively.

Fluorescence Quenching Measurements

Fluorometric titrations of purified anti-heptapeptide antibody with myoglobin and the N^{α} DNP-heptapeptide derivative were carried out using an Aminco-Bowman spectrophotofluorometer according to Velick et al. (266). All protein and peptide solutions were prepared with PBS** and were passed through a millipore filter (0.45 μ porosity) just prior to the fluorescence measurement to ensure removal of any insoluble material.

Samples of purified antibody (2 ml) in the concentration range 3×10^{-7} to 6×10^{-7} M were accurately introduced into a 1 cm^2 quartz cuvette contained in a chamber maintained at 25°, and allowed to equilibrate for about 10 minutes. The antibodies

---

*The molar extinction coefficient of N^{α} DNP-heptapeptide, measured at 360 nm, was determined to be 17,000 by Dr. J. Diment (unpublished results).

**For all fluorescence measurements doubly-distilled water was used to prepare the buffer, and all glassware used for handling the solutions was cleaned in concentrated nitric acid.

were excited at 287 nm, and the initial fluorescence at the appropriate wavelength (depending on the titrant) was recorded. Small aliquots (0.001-0.005 ml) of the hapten or myoglobin solution were then added by means of an 'Agla' micrometer syringe. After each addition the solution was stirred gently by means of a platinum coil (which remained in the solution during the titration), and the fluorescence level recorded after a fixed interval of 2 minutes. Titrations were carried out in duplicate. To correct for non-specific quenching and titrant attenuation, control titrations were carried out with normal RGG at the same concentration as the antibody solution.

The emitted fluorescence intensity observed was corrected for solvent blank which was deducted from each reading. A correction for dilution was made and the corrected fluorescence intensity was then plotted as a function of ligand concentration.

Prior to titrating with myoglobin, the absorption spectrum of myoglobin was determined spectrophotometrically and compared with the emission spectrum of the antibodies. For titrations with myoglobin the emission was measured at 350 nm in some experiments while in others it was measured at 388 nm, which corresponds to the wavelength at which the antibody emission curve and myoglobin absorption curve intersect.

RESULTS

Elution of Antibodies from Immunosorbents

(i) BAC-H

Rabbit anti-myoglobin antiserum (12 ml) was incubated with BAC-H and eluted sequentially with a total of 40 μmole each of tetra-, penta-, hexa-, and hepta- peptides (in that order), and finally with glycine-HCl, pH 2.5. The results are shown in Figure 8. The tetra- and penta- peptides, at the concentration used here, were ineffective in eluting material from the heptapeptide immunosorbent. A small amount of material was removed with the hexapeptide and the major portion of antibody was eluted with the heptapeptide. Subsequent treatment with glycine-HCl, pH 2.5 revealed no antibody in the eluate. Subsequent elution with glycine-HCl, pH 2.0, also revealed no protein in the eluate, ruling out the possibility of high affinity antibody still attached to the immunosorbent. None of the eluates gave precipitin bands on immunodiffusion against myoglobin, however, the absorbed serum still showed a band, indicating the presence of residual antibodies to other determinants of myoglobin.

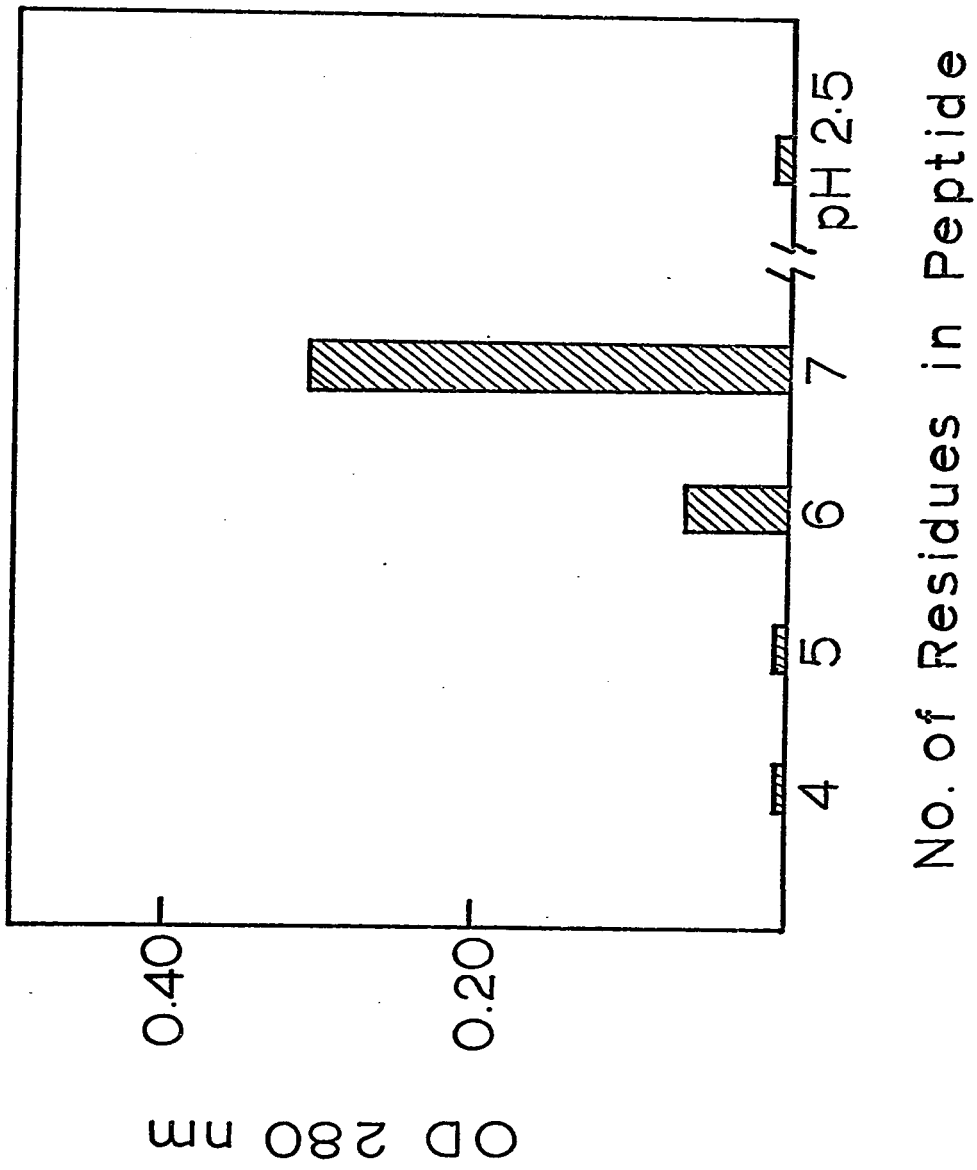
When BAC-H was eluted directly with glycine-HCl, pH 2.5, all the antibody appeared to be released since subsequent treatment at pH 2.0 did not result in further elution of protein. No precipitin bands were formed when the acid eluates from BAC-H were diffused against myoglobin.

(ii) BAC-Mb

BAC-Mb was incubated with rabbit anti-myoglobin antiserum

Figure 8

Sequential elution of antibody from BAC-H with peptides. The C-terminal tetra-, penta-, hexa-, and hepta-peptides were used individually with the immunosorbent in order of increasing chain length. After elution with the hepta-peptide the immunosorbent was eluted with glycine-HCl, pH 2.5.


OD 280 nm
0.40
0.20
4
5
6
7
pH 2.5
No. of Residues in Peptide

(12 ml) and eluted sequentially in the same way as BAC-H. The results are shown in Figure 9. In this case, all the peptides showed some ability to elute antibodies. However, again, the heptapeptide appeared to be the most effective in releasing antibodies from the immunosorbent. None of the eluates nor the absorbed antiserum formed precipitin bands on immunodiffusion against myoglobin.

In some cases, the antibody was eluted directly with glycine-HCl instead of with peptides. Elution of BAC-Mb with glycine-HCl, pH 2.5, resulted in the release of about 70-80% of the total antimyoglobin antibodies. The remaining 20-30% could be eluted only when the pH was lowered to 2.0. Both eluates gave precipitation bands on immunodiffusion against myoglobin.

Anti-myoglobin serum (25 ml) was incubated with BAC-Mb and eluted with the tetradecapeptide (total of 40 μmole). After exhaustive dialysis the $O.D._{280}$ of the 2.0 ml concentrate was 0.41. The eluate did not give a precipitin band on immunodiffusion with myoglobin.

Radioimmunodiffusion

The results of the radioimmunodiffusion are shown in Figure 10. All of the eluates tested reacted specifically with radioiodinated myoglobin, as revealed by the presence of bands on the X-ray film (Figure 10 B). However, although the control RGG showed the formation of a precipitin band (Figure 10 A), no corresponding radioactive band was visible on the film.

Figure 9

Sequential elution of antibody from BAC-Mb with peptides. The C-terminal tetra-, penta-, hexa-, and hepta- peptides were used individually with the immunosorbent in order of increasing chain length.

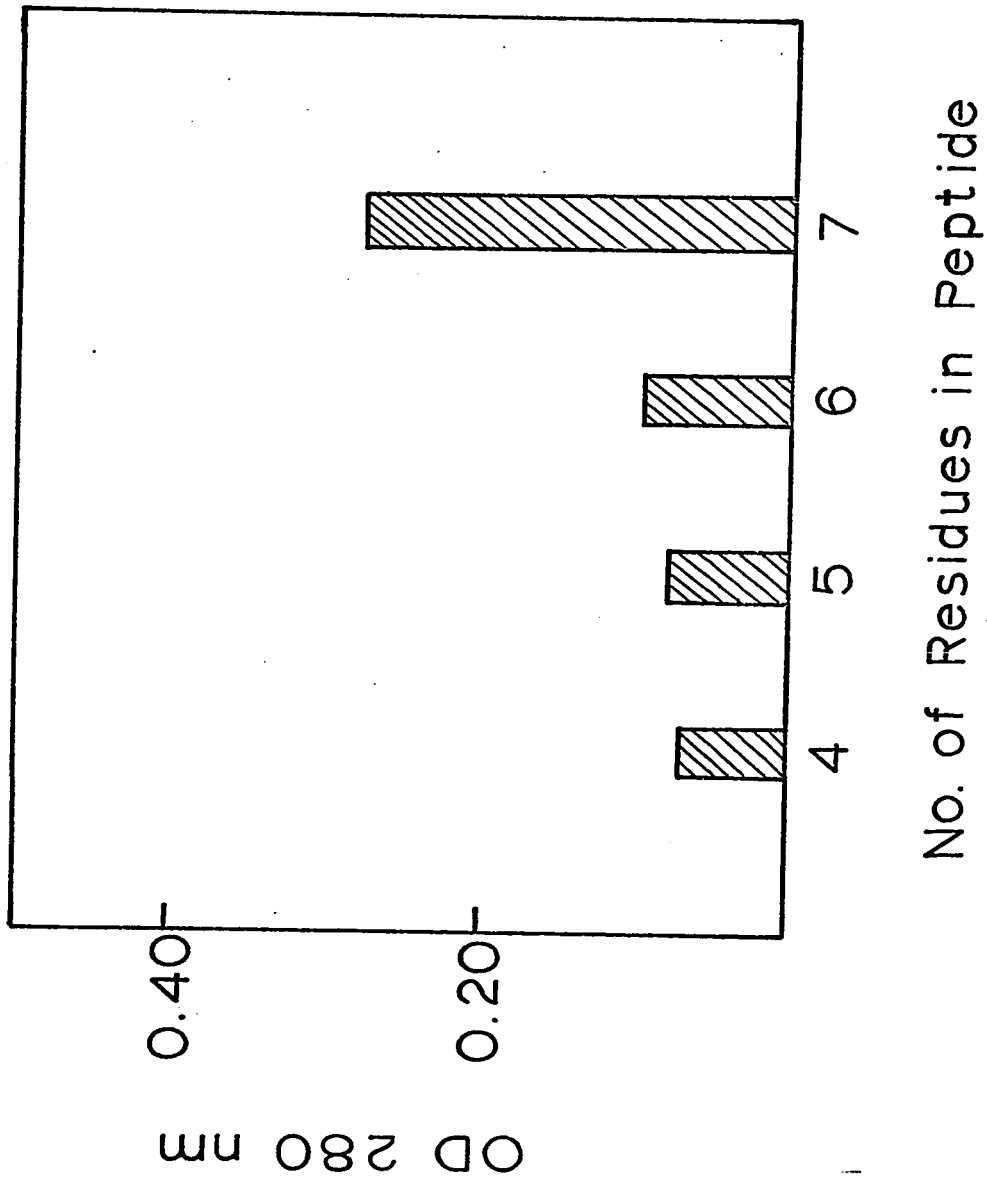
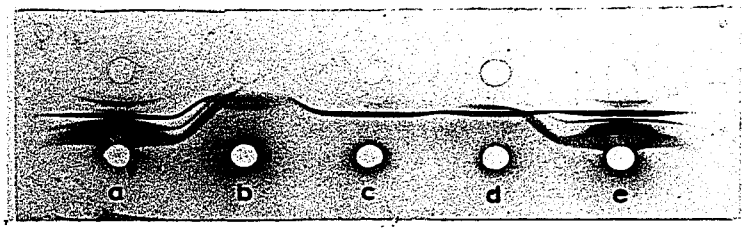

OD 280 nm
0.40
0.20
4
5
6
7
No. of Residues in Peptide

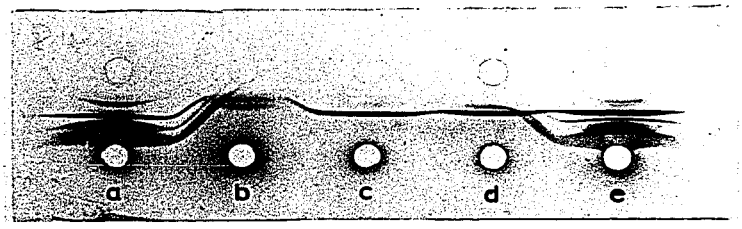
Figure 10

Radioimmunodiffusion of purified antibody. Samples were mixed with RGG and diffused against sheep anti-rabbit serum, followed by diffusion of ^{125}I-myoglobin. (A) immunodiffusion pattern in agar; (B) radioautographic bands on X-ray film. (a) eluate from BAC-Mb at pH 2.5; (b) eluate from BAC-Mb at pH 2.0; (c) tetradecapeptide eluate from BAC-Mb; (d) heptapeptide eluate from BAC-H; (e) RGG control.



A

B



A

B

Disc Electrophoresis

The results of the electrophoresis in polyacrylamide gel are shown in Figure 11. The heptapeptide eluate from BAC-H (Figure 11 A) revealed a more limited heterogeneity when compared with the acid eluate from BAC-Mb (Figure 11 B), covering a very narrow range of the electrophoretic mobilities of γ-globulin. In addition, a band corresponding to that of IgM globulin is visible as well as a trace of albumin.

The amount of antibody eluted from BAC-Mb at pH 2.0 is much less than that eluted at pH 2.5 (Figure 11 B), and the pH 2.0 eluate appears to have a more restricted heterogeneity than the pH 2.5 eluate.

Ultracentrifugal Analysis

The antibody eluted from BAC-H was ultracentrifugally heterogeneous containing two components (Figure 12). The corrected S_{20} values of the slower and faster-moving components were 5.8 and 16.5 respectively, corresponding to IgG and IgM antibodies.

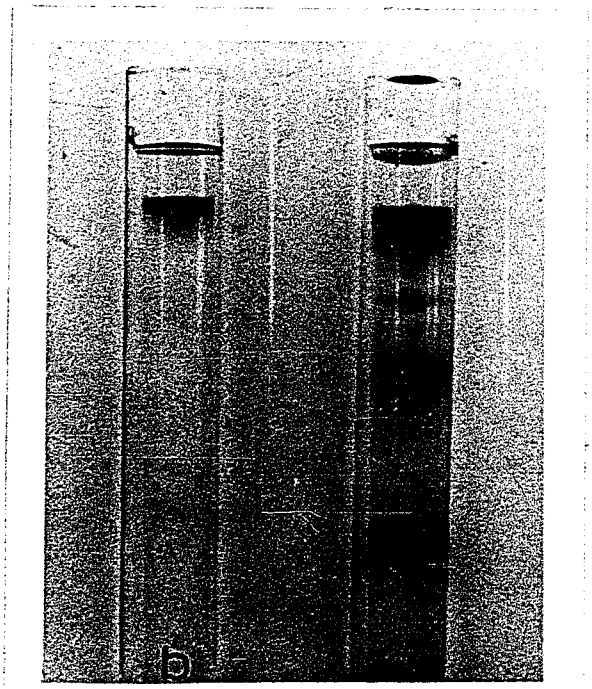
Fluorescence Quenching

The quenching curve for the titration of anti-heptapeptide antibodies with N^{α} DNP-heptapeptide is shown in Figure 13. When the curve of the antibody titration is compared with that of the non-specific RGG it is evident that very little, if any, specific quenching due to antibody-hapten complexing could be measured.

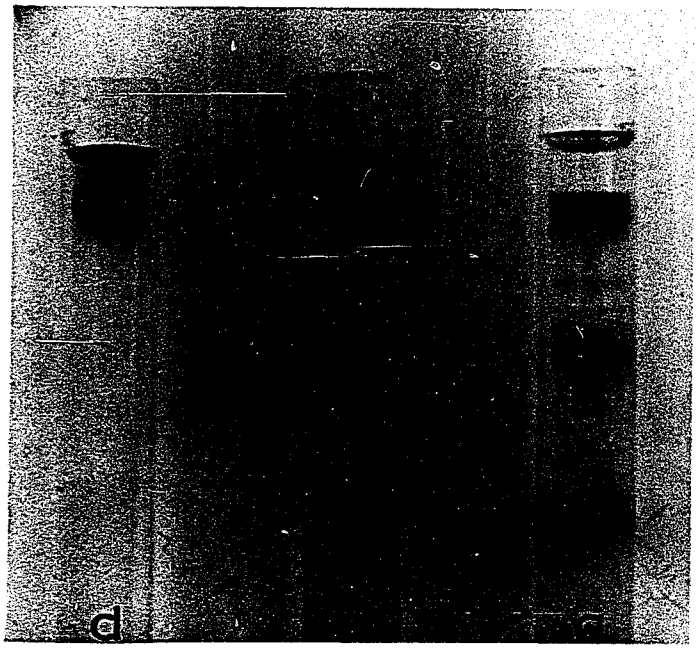
The antibody emission and myoglobin absorption spectra are shown in Figure 14. The antibody fluorescence was excited

Figure 11

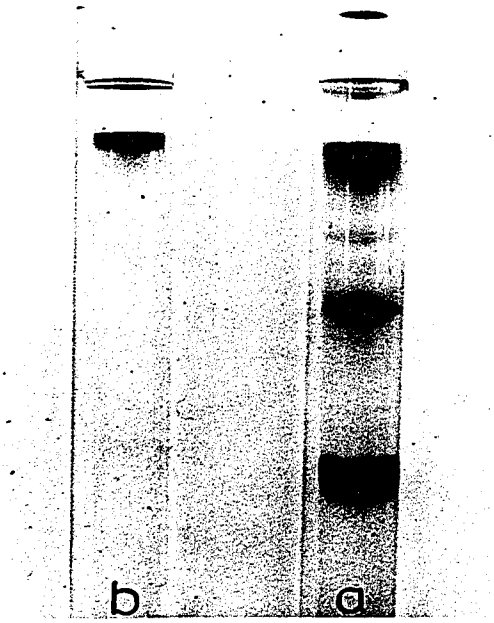
Disc electrophoretic patterns of purified antibody eluted from (A) BAC-H and (B) BAC-Mb. (a) whole rabbit anti-myoglobin serum; (b) heptapeptide eluate from BAC-H; (c) eluate from BAC-Mb at pH 2.0; (d) eluate from BAC-Mb at pH 2.5.



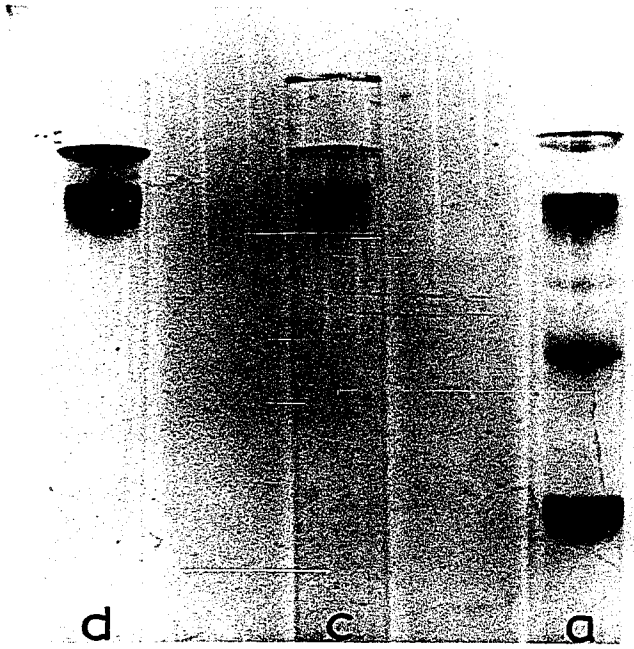
A



B



A



B

Figure 12

Ultracentrifugal pattern of the antibody eluted from BAC-H. The run was performed in a Spinco Model E ultracentrifuge using a synthetic boundary cell. The analysis was carried out in PBS at 20° and 59,780 rpm. Photographs were taken every four minutes after full speed was attained. Sedimentation is from left to right.

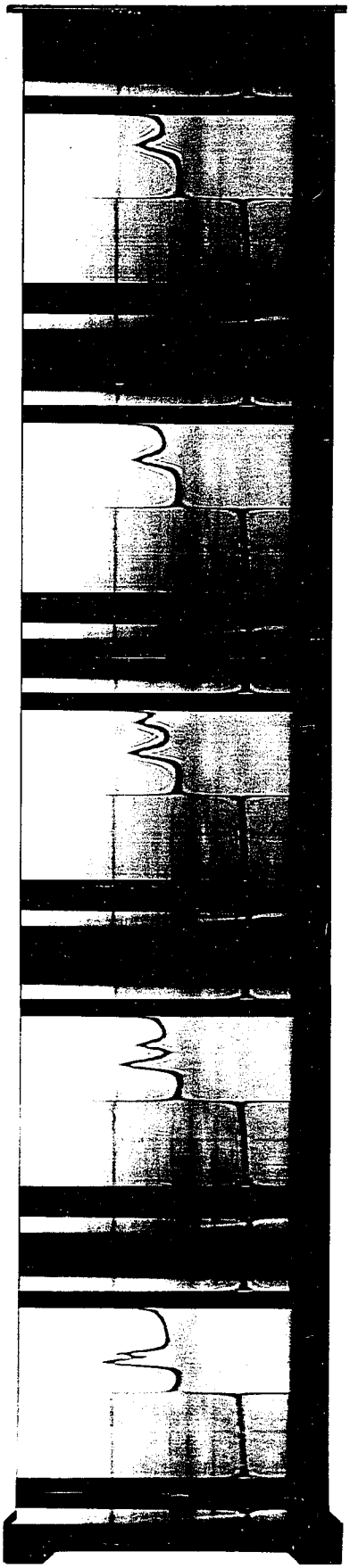


Figure 13

Typical quenching curves for the titration of (A) anti-heptapeptide antibody (3.6×10^{-7} M) and (B) RGG (3.6×10^{-7} M) with N^{α} DNP-heptapeptide (4.4×10^{-5} M). λ_{ex} = 287 nm, λ_{em} = 350 nm.

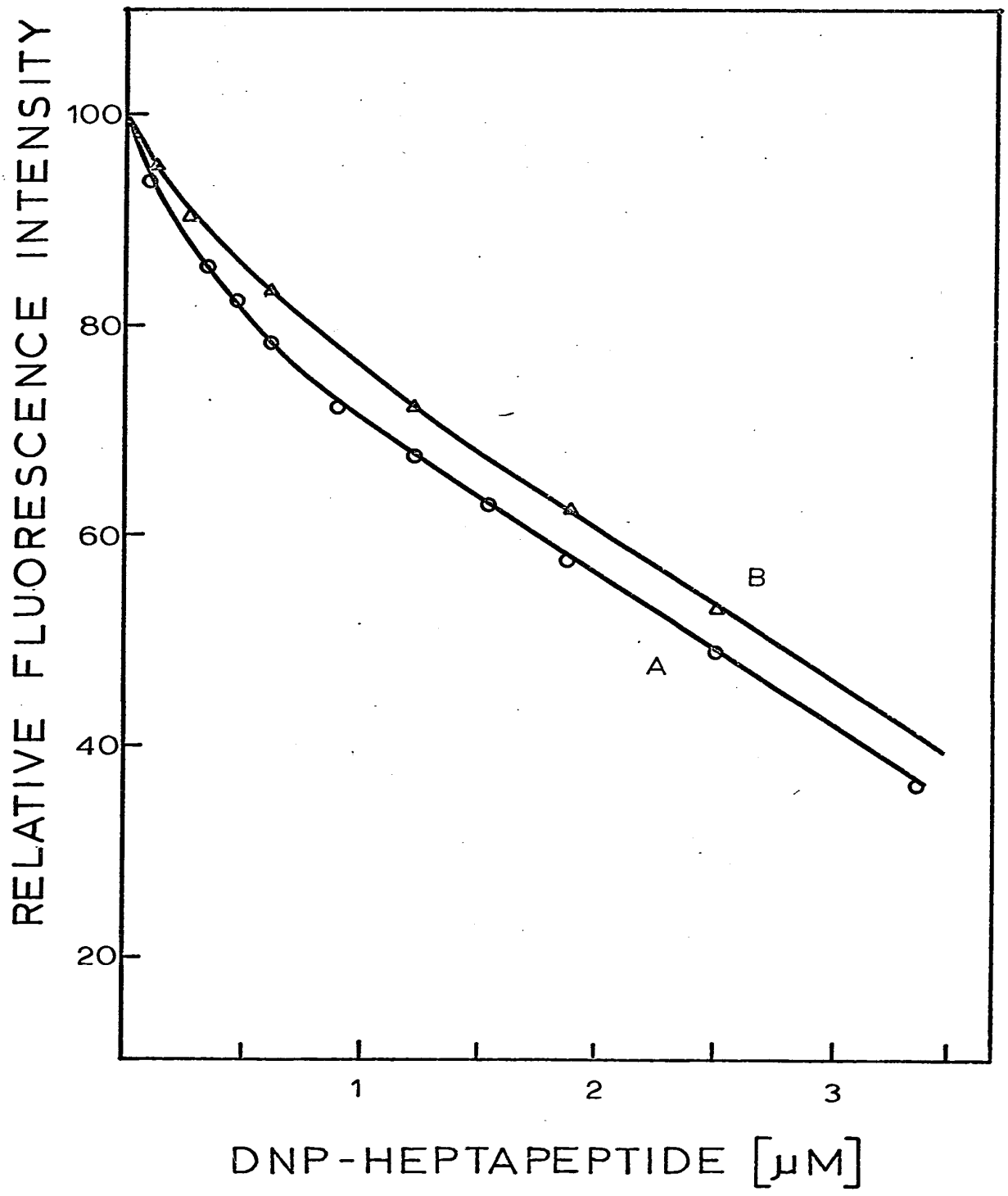
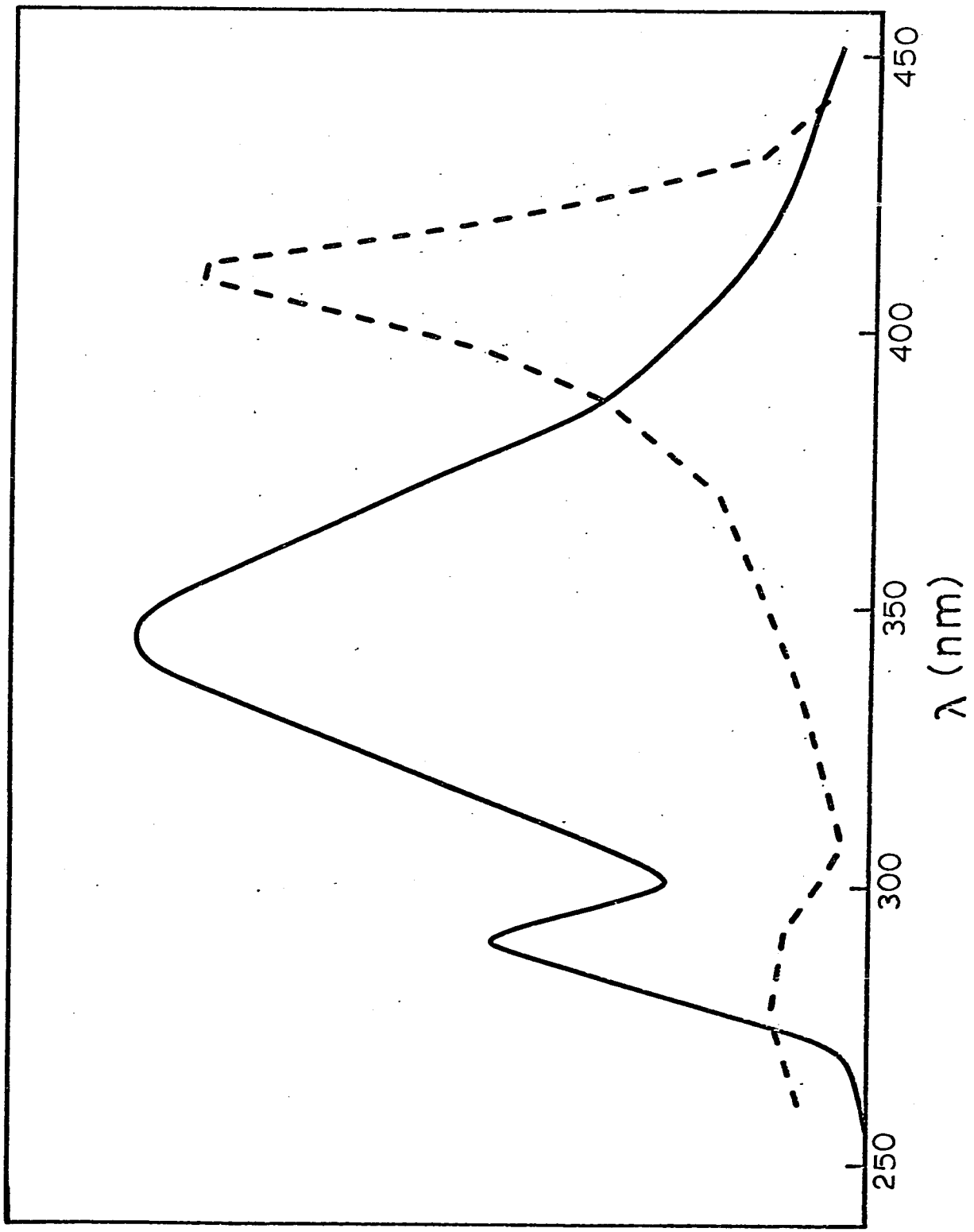

RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
1
2
3
DNP-HEPTAPEPTIDE [μM]
A
B

Figure 14

Antibody emission (——) and myoglobin absorption (---) spectra.


450
400
350
300
250
λ (nm)

at 287 nm, corresponding to its absorption maximum. As can be seen, the maximum fluorescence emission of the antibody solution occurred at 345 nm while the maximum of the absorption peak of myoglobin occurred at 409 nm. Some degree of overlap is evident - the point of intersection occurring at 388 nm, at which wavelength the fluorescence in some experiments was measured.

The quenching curves for the titration of antibodies with myoglobin at 350 nm is shown in Figure 15, and that measured at 388 nm is shown in Figure 16.

As can be seen, in no case was there any significant specific quenching. Also, the ligand attenuation is quite large. Because of the lack of significant quenching and the large degree of attenuation, no attempt was made to calculate the maximum quenching (Q_{max}) nor the intrinsic binding constant, K_o.

Figure 15

Typical quenching curves for the titration of (A) anti-heptapeptide antibodies (3.6×10^{-7} M) and (B) RGG (3.6×10^{-7} M) with myoglobin (9.1×10^{-5} M). λ_{ex} = 287 nm, λ_{em} = 350 nm.

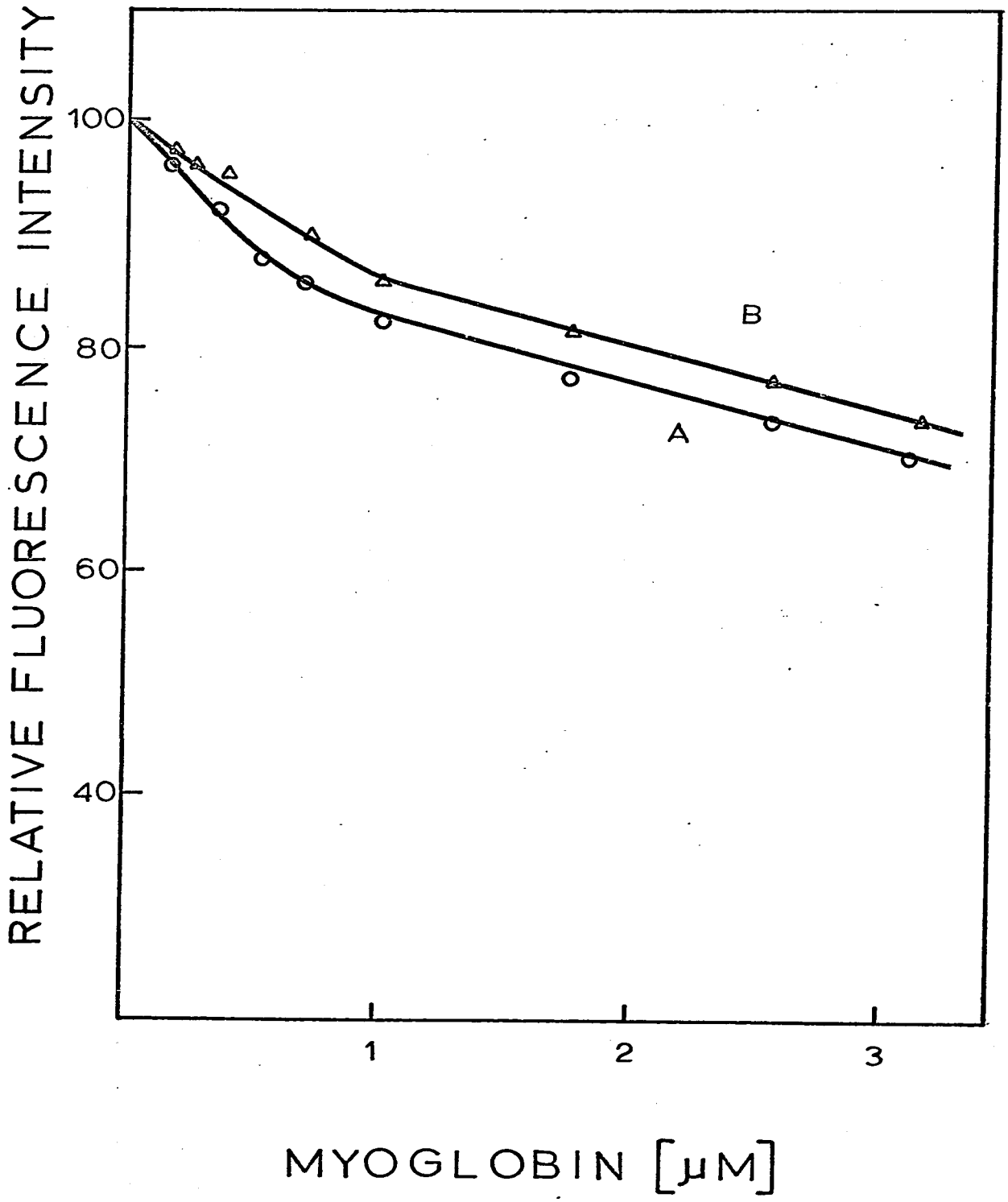
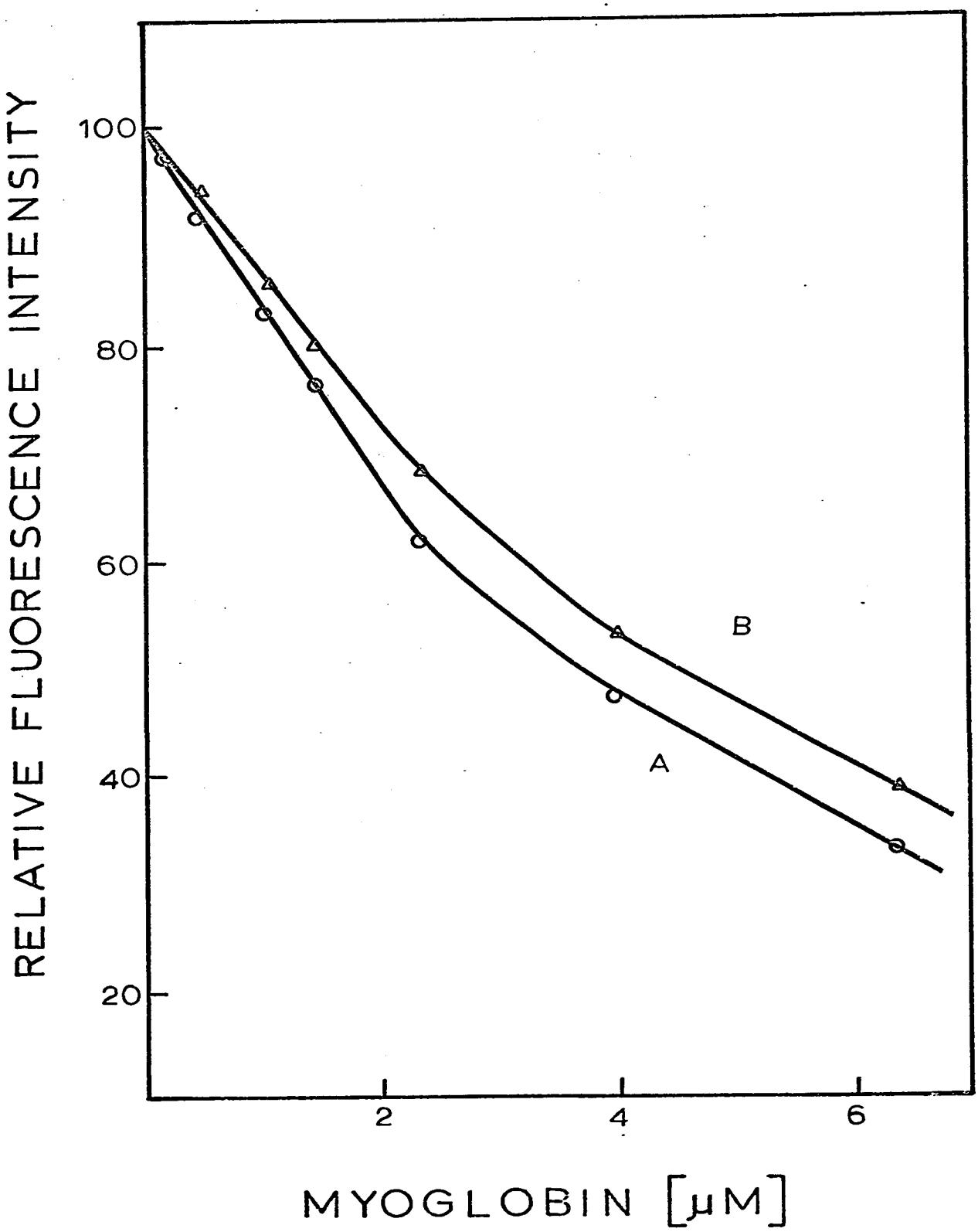

RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
1
2
3
B
A
MYOGLOBIN [μM]

Figure 16

Typical quenching curves for the titration of (A) anti-heptapeptide antibodies (6.0×10^{-7} M) and (B) RGG (6.0×10^{-7} M) with myoglobin (9.1×10^{-5} M). λ_{ex} = 287 nm, λ_{em} = 388 nm.


RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
2
4
6
MYOGLOBIN [μM]
A
B

DISCUSSION

Preparation of Immunosorbents

The Bac-H and BAC-Mb immunosorbents were prepared with relative ease and simplicity. Nevertheless, their preparation was not as simple as that of the ethylene-maleic anhydride-myoglobin (EMA-Mb) immunosorbent which had been used in a previous study (129). However, in spite of this EMA-Mb was abandoned in favor of BAC-Mb for two reasons: (i) because of its rheological properties, EMA-Mb could not be used in batchwise operations, which was the method of choice in this study; BAC-Mb is easily handled in this type of operation; (ii) EMA is a polyanion in aqueous solution and may act as an ion exchange resin, adsorbing proteins from serum non-specifically, although there is no evidence that this occurred with the myoglobin system. The use of EMA with the heptapeptide coupled to it was impossible since with this small molecule insufficient cross-linking occurred during coupling and therefore the resulting conjugate remained soluble.

Bromoacetylcellulose (BAC) immunosorbents could be used with small quantities of sera and peptides and they are stable over long periods of time and could be used repeatedly.

No attempt was made to determine the extent of coupling of the myoglobin and heptapeptide to BAC. But visual comparison of the myoglobin solution before and after coupling showed that the dark brown color of myoglobin had been transferred from the solution to the insoluble BAC, indicating an almost quantitative coupling of myoglobin. No such visual aid was possible with

the heptapeptide, nor were spectrophotometric measurements attempted.

Isolation and Identification of Antibodies

The isolation of homologous antibodies directed against a single antigenic determinant from an appropriate immunosorbent using a synthetically prepared antigenic determinant as a hapten can be accomplished with relative simplicity. The fact that the radio-labeled myoglobin was incorporated into precipitates formed by eluted antibodies and sheep anti-rabbit serum indicates that the eluted material consisted of antibodies directed against determinants of myoglobin. It is highly significant that no incorporation was evident in the precipitates formed by normal RGG used as a control. Hence, the bands on the X-ray films were not due to non-specific occlusion or adsorption, but to specific combination of the labeled myoglobin with eluted antibodies. The fact that, in agar gel, the purified antibodies alone did not give a precipitate with myoglobin is considered as evidence that they were directed solely against the univalent antigenic determinant corresponding to the C-terminal hepta-peptide of myoglobin in one instance, and to the region consisting of residues 56-69 of myoglobin in the other case.

The anti-heptapeptide antibodies consisted of a restricted population of antibodies, possessing a very narrow range of electrophoretic mobilities in polyacrylamide gel at pH 8.3. These results may not seem to agree with the previous claim that electrophoretically homogeneous antibodies had been isolated. This apparent discrepancy can be explained by

pointing out that, in the former study, the eluted antibodies had been dialyzed against water prior to electrophoresis, a procedure which would be expected to remove the euglobin fraction. Furthermore, in the previous study, the amount of material isolated was too small for ultracentrifugal analysis and therefore it is possible that, in spite of apparent electrophoretic homogeneity, the antibodies may have been ultracentrifugally heterogeneous, as they were in the present study.

It must be stressed that no attempt was made in this study to quantitatively determine the amount of antibody adsorbed to the immunosorbents. However, the fact that antiserum absorbed with BAC-Mb did not give precipitin bands with myoglobin indicates that, at least in this case, the antibodies were quantitatively adsorbed. In view of the small quantities of antibodies available, no quantitative precipitin tests were performed on the original sera to determine their antibody content.

The results of the sequential elution of antibodies with the C-terminal peptides will be discussed in the next chapter.

Fluorescence Quenching

(i) Rationale of this study

The C-terminal heptapeptide and three of its lower homologs were synthesized with a view to establishing their usefulness as probes for the more precise determination of the antigenic activity of this region of myoglobin. Since the purified antibodies to this determinant bind the heptapeptide to form soluble complexes it was felt that fluorescence quenching might provide a method for quantitatively measuring such binding.

One very serious drawback, however, was the poor overlap between the absorption spectrum of the hapten (the peptide) and the fluorescence emission spectrum of the antibodies. In an effort to overcome this difficulty, a DNP group was attached to the alpha amino group of the heptapeptide in the hope that, in spite of the alteration of the hapten, significant energy transfer would occur so as to make binding measurements possible. The alpha, rather than the epsilon, amino group of the lysine was chosen for substitution since, in the native molecule, this group is involved in a peptide bond with the neighboring amino acid in the protein chain. Hence, substitution in this position would make a less drastic change in the structure of the hapten than would substitution of the side-chain amino group, and therefore, binding with antibody would be less affected. It was also hoped that there were tryptophan residues within, or sufficiently close to, the binding site of the antibody for energy transfer to occur.

In addition, it has been found that when antibody combines with a hemoprotein antigen, the fluorescence of the tryptophan residues of the antibody is partially quenched (277). The suggested reason for this is that the heme prosthetic group can act as an acceptor in the energy transfer process. On this basis, it was reasoned that the heme group in myoglobin might also serve to quench the fluorescence of the antibody tryptophan residues. Hence, binding studies of myoglobin and purified antibodies directed to the C-terminal heptapeptide were undertaken. In this way it was hoped to be able to

compare the binding of the C-terminal peptides with that of the intact antigen.

(ii) Discussion of results

The experiments reported here indicate that the fluorescence of the anti-heptapeptide antibodies was not quenched significantly by the DNP-heptapeptide nor by myoglobin. There are several possible explanations for this:

(a) The spectral conditions for radiationless energy transfer from antibody to antigen (myoglobin) were not met, i.e., there was not sufficient overlap of the antibody fluorescence spectrum by the absorption spectrum of the antigen (see Figure 14).

(b) In the case of the reaction of antibody with N^{α} DNP-heptapeptide, although the heptapeptide may have been strongly bound by the antibody, the acceptor DNP group most probably was beyond the effective interaction distance, R_o, for energy transfer to occur. R_o is a function of the orientations of the two species, i.e., the primary tryptophan oscillators and the recipient DNP group. Since energy transfer occurs through dipole-dipole coupling and not through orbital overlap, it is highly probable that the two species were not dipole-coupled through the heptapeptide chain.

It is also possible that the DNP substituent altered the structure of the heptapeptide in such a way as to make binding with antibody impossible. From these experiments, it was not possible to deduce if binding occurred at all between antibodies and the DNP-heptapeptide.

CHAPTER IV

GENERAL DISCUSSION

In the present study an attempt was made to arrive at a more precise definition of the antigenic determinant(s) contained in the C-terminal heptapeptide of sperm-whale myoglobin. The C-terminal tetra- and penta- peptides were ineffective in removing antibody bound to the heptapeptide immunosorbent, indicating that the residues up to and including the penta-peptide, Leu-Gly-Tyr-Gln-Gly, do not possess sufficient binding energy to remove antibodies bound to the BAC-H immunosorbent. However, the addition of the sixth residue, glutamic acid, appears to provide the necessary binding energy for the release of at least a small portion of the antibodies. Furthermore, with the addition of the final residue, lysine, the binding energy became sufficiently large to effect the removal of all the bound antibodies, as evidenced by the fact that no antibody could subsequently be eluted at pH 2.5 or at pH 2.0. In these experiments, no attempt was made to establish if the antibodies eluted with the hexa- and hepta- peptides represented different populations of antibodies whose antibody combining sites differed from each other, or if, in fact, they represented the same population of antibodies, a fraction of which could be removed by the hexapeptide at the particular concentration used here; i.e., it is possible that at a larger concentration the hexapeptide may have been able to elute the remaining antibody as well.

It seems evident that BAC-H was not effective in binding all the antibodies directed against the C-terminal heptapeptide since the tetra- and penta-peptides, which did not elute antibodies off BAC-H, were capable of eluting a small portion of the antibodies bound to BAC-Mb. It is possible that steric factors may have been responsible for the inability of some antibodies to bind to BAC-H. However, the results obtained with both immunosorbents indicate that the N-terminal lysine residue of the heptapeptide does indeed play a major role in the binding between this peptide and the corresponding antibodies.

These results contrast somewhat with those reported by Crumpton et al. (130), who showed that the hexa- and hepta-peptides possessed the same activity as measured by inhibition of precipitation. This discrepancy may be ascribed to the different methods used to measure binding activity or to the different antisera used in the two studies. It should also be noted that these authors used whole serum to measure binding rather than purified antibodies. It is conceivable that the serum may have contained peptidases which may have altered the heptapeptide and hence reduced its activity. Such peptidases have been found to interfere in inhibition studies with peptides (102). In addition, it was also reported (130) that the tetra- and penta- peptides possessed a small amount of inhibitory activity. This latter finding is in agreement with the results of this study, i.e., that these smaller peptides were capable of eluting antibodies from the BAC-Mb immunosorbent. It must be mentioned that Atassi too, found some activity

associated with the C-terminal hexapeptide (117), but only to the extent of 11% (with apomyoglobin) as compared with 15% (with apomyoglobin) for the heptapeptide (68). However, a comparison of activities obtained from different studies is not entirely valid since different sera behave in different ways with the same peptide.

An examination of the 3-dimensional model of myoglobin (80) reveals that the N-terminal lysine residue of the heptapeptide (lysine-147) is an exposed residue. Therefore, there seems to be no evident reason why this residue would not form part of the C-terminal antigenic site. Indeed, the results reported here show that it does contribute to this site. In addition, nitration of tyrosines 146 and 151 (Figure 2, Table I) caused a complete loss in the activity of peptide 132-153 (127), indicating that one or both of these residues are present in a reactive region of myoglobin. However, replacement of tyrosine-151 with phenylalanine or p-methoxyphenylalanine (130) did not alter the activity of this region. These results may indicate that tyrosine-146 forms part of the antigenic site and that it is the C-terminal portion of peptide 139-146 and the N-terminal portion of peptide 147-153, which together, constitute the antigenic determinant.

Some evidence has been obtained which indicates that the C-terminal dipeptide of myoglobin may not be necessary for activity (118). If this is the case, then it does not seem unreasonable to propose that the N-terminus of peptide 147-153 is important in binding with antibody. However, it should be

mentioned that peptide 147-151 possessed less than half the activity of the C-terminal heptapeptide, 147-153 (128). It has been suggested that the C-terminal dipeptide is not actually a 'contact' point with antibody but only serves to orientate the C-terminal heptapeptide into a more favorable conformation for binding with antibody (131).

The fact that lysine-147 has been shown to play an important role in the binding of the C-terminal heptapeptide to antibody does not necessarily mean that this residue is the immunodominant group (278) of this antigenic determinant, i.e., it may not be the residue which contributes the highest proportion of the binding energy. In this regard, the studies with N^{α} DNP-oligolysines are of interest (106). On a molar basis, maximum inhibition of precipitation was obtained with the heptalysine derivative. However, when the differences in binding energy for the N^{α} DNP-oligolysyl peptides relative to the heptamer were computed, it was found that as the size of the oligopeptide decreased the binding energy was progressively reduced. But with change from the heptamer to the trimer, the decrease in energy was comparatively small, suggesting that the trimer contributed the major portion of binding (92%) and that the additional lysine residues, up to the heptamer, made only a small contribution. A similar situation may exist with the C-terminal region of myoglobin. The actual binding energy contributed by lysine-147 may be relatively small. However, without the additional increment provided by this residue the cumulative binding energies of the other residues may be

insufficient to elute antibody from an immunosorbent. The actual immunodominant group of this antigenic determinant can be determined only by quantitative binding studies.

The results of the electrophoretic and ultracentrifugal analyses indicate that the eluate from the BAC-H immunosorbent represented a population of antibodies with restricted heterogeneity. Hence, it can be seen that the antibody response, even to a single small, relatively simple, naturally occurring protein antigenic determinant, is a complex one. In connection with this, it is interesting to note that antibodies directed against the 'loop' peptide of lysozyme also possessed a limited heterogeneity as determined by polyacrylamide gel electrophoresis and isoelectric focusing (92). Nor is the lack of homogeneity of antibodies directed to the C-terminal heptapeptide of myoglobin limited to rabbit antisera. Horse antibodies, specifically eluted with the heptapeptide from an ethylene-maleic anhydride-myoglobin immunosorbent, revealed the presence of three components when electrophoresed on polyacrylamide gel (279).

In the present study, some anti-myoglobin antibodies may have possessed very high affinity for the antigen since they could be dissociated from BAC-Mb only at pH 2.0; this fraction may have consisted of conformation-dependent antibodies. It does not seem, however, that the antibodies bound to the BAC-H immunosorbent belonged to this group since all the antibodies were released from this immunosorbent at pH 2.5 and subsequent treatment with buffer at pH 2.0 did not result in further elution

of any antibody. In the myoglobin molecule the C-terminal heptapeptide may possess a certain conformation which would be stabilized by the rest of the molecule and with which the conformation dependent antibodies could combine. This conformation would, of course, be lacking in BAC-H, i.e, these antibodies either would not combine or, if they did, might be more easily removed.

The antibodies directed against the tetradecapeptide are an interesting subject for further study since, while this peptide has little or no helical conformation (123), it represents a portion of myoglobin which in the native molecule is an essentially helical region (80). Yet the antibodies directed against the helical antigenic determinant still combined with the free non-helical peptide.

In summary, it has been shown that by sequential elution of antibodies bound to immunosorbents, with a series of synthetic peptides related to the C-terminal heptapeptide of myoglobin, in order of their increasing chain lengths, the N-terminal lysine residue of the heptapeptide plays an important role in the binding of myoglobin with the antibodies directed to the C-terminus of myoglobin. The isolated antibodies possessed a limited heterogeneity electrophoretically and ultracentrifugally. In addition, a synthetic tetradecapeptide, corresponding to the helical region in myoglobin (residues 56-69), was shown to possess antigenic activity.

CLAIMS TO ORIGINALITY

The heptapeptide, Lys-Glu-Leu-Gly-Tyr-Gln-Gly, representing the C-terminus of sperm-whale myoglobin, and the hexa-, penta-, and tetra- peptide homologs of this heptapeptide, having sequences, Glu-Leu-Gly-Tyr-Gln-Gly, Leu-Gly-Tyr-Gln-Gly, and Gly-Tyr-Gln-Gly, respectively, were synthesized by the Merrifield method of solid-phase synthesis. Immunosorbents, prepared by coupling (i) the heptapeptide, or (ii) myoglobin, to bromoacetylcellulose, were used to isolate antibodies directed to the heptapeptide from rabbit anti-myoglobin sera. It was found that the heptapeptide was most effective in eluting antibodies from either immunosorbent, pointing to the presence of the N-terminal lysine as an important factor in the binding between the heptapeptide and the corresponding antibodies.

Disc electrophoresis revealed that these isolated antibodies had a limited heterogeneity, possessing a very narrow range of electrophoretic mobilities. These antibodies were also ultra-centrifugally heterogeneous, consisting of two components.

Using the technique of fluorescence quenching, an attempt was made to measure binding between the isolated antibodies and (i) myoglobin, or (ii) N^{α} DNP-heptapeptide, which was synthesized by the solid-phase technique.

In addition, the tetradecapeptide, Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly-Val-Thr-Val-Leu, corresponding to the helical region of myoglobin, residues 56-69, was synthesized by the solid-phase method and the corresponding antibodies were isolated from the myoglobin immunosorbent.

REFERENCES

1. Boyd, W. Fundamentals of Immunology, 4th edition, Interscience Publishers, New York (1966).

2. Landsteiner, K. The Specificity of Serological Reactions, Revised ed., Dover Publications Inc., New York (1945).

3. Sehon, A.H. Encyclopedia of Polymer Science and Technology, (H.F. Marek, N.M. Bikales and N.G. Gaylord, eds.), Interscience Publishers, New York, Vol. 2 (1964).

4. Landsteiner, K. and Vander Scheer, J. J. Exp. Med. 50, 407 (1929).

5. Boyd, W.C. Introduction to Immunochemical Specificity, Interscience Publishers, New York (1962).

6. Marrack, R.J. The Chemistry of Antigens and Antibodies, Report no. 230, Medical Research Council, H.M.S.O., London (1938).

7. Pauling, L. J. Amer. Chem. Soc. 62, 2643 (1940).

8. Eisen, H.N. and Karush, F. J. Amer. Chem. Soc. 71, 363 (1949).

9. Singer, S.J. and Campbell, D.H. J. Amer. Chem. Soc. 75, 5577 (1953).

10. Day, E.D. Foundations of Immunochemistry, The Williams and Wilkins Co., Baltimore, Md. (1966).

11. Pepe, F.A. and Singer, S.J. J. Amer. Chem. Soc. 81, 3878 (1959).

12. Kleczkowski, A. Immunol. 8, 170 (1965).

13. Najjar, V.A. and Fisher, J. Science 122, 1272 (1955).

14. Harshman, S., Robinson, J.P. and Najjar, V.A. Ann. N.Y. Acad. Sci. 103, 688 (1963).

15. Najjar, V.A. Physiol. Rev. 43, 243 (1963).

16. Hurwitz, E., Fuchs, S. and Sela, M. Biochim. Biophys. Acta 111, 512 (1965).

17. Najjar, V.A., Robinson, J.P., Lawton, A.R. and Fidalgo, B.V. Johns Hopkins Medical J. 120, 63 (1967).

18. Crumpton, M.J. Biochem. J. 100, 223 (1966).

19. Ouchterlony, O. Act. Path. Microbiol. Scand. 26, 507 (1949).

20. Boyden, S.V. J. Exp. Med. 93, 107 (1951).

21. Gordon, J., Rose, B. and Sehon, A.H. J. Exp. Med. 108, 37 (1958).

22. Sehon, A.H. Mechanisms of Hypersensitivity, (J.H. Shaffer, G.A. LoGrippo and M.W. Chase, eds.), Little, Brown and Co., Boston, Mass. (1959).

23. Jerne, N.K. and Avegno, P. J. Immunol. 76, 200 (1956).

24. Makela, O. Immunol. 10, 81 (1966).

25. Haimovitch, J. and Sela, M. J. Immunol. 97, 338 (1966).

26. Carter, B.G., Yo, S.L. and Sehon, A.H. Can. J. Biochem. 46, 261 (1968).

27. Hornick, C.L. and Karush, F. Israel J. Med. Sci. 5, 163 (1969).

28. Sela, M. Adv. Immunol. 5, 30 (1966).

29. Sela, M. Bull. Soc. Chim. Biol. 46, 1685 (1964).

30. Sela, M., Fuchs, S. and Arnon, R. Biochem. J. 85, 223 (1962).

31. Gilliland, P.F. and Prout, T.E. Metabolism 14, 918 (1965).

32. Roth, J., Glick, S.M., Klein, L.A. and Peterson, M.J. J. Clin. Endocrinol. 26, 671 (1966).

33. Dietrich, F.M. Int. Arch. Allergy 30, 497 (1966).

34. Schlossman, S.F., Yaron, A., Ben-Efraim, S. and Sober, H.A. Biochem. 4, 1638 (1965).

35. Borek, F., Stupp, Y. and Sela, M. J. Immunol. 98, 739 (1967).

36. Leskowitz, S., Jones, V.E. and Zak, S.J. J. Exp. Med. 123, 229 (1966).

37. Spitler, L., Benjamini, E., Young, J.D., Kaplan, H. and Fudenberg, H.H. J. Exp. Med. 131, 133 (1970).

38. Jaton, J.-C. and Sela, M. J. Biol. Chem. 243, 5616 (1968).

39. Gill, T.J., Kunz, H.W. and Papermaster, D.S. J. Biol. Chem. 242, 3308 (1967).

40. Gill, T.J., Papermaster, D.S. and Mowbray, J.F. Nature 203, 644 (1964).

41. Janeway, C.A. and Humphrey, J.H. Immunol. 14, 225 (1968).

42. Sela, M. and Mozes, E. Proc. Nat. Acad. Sci. U.S.A. 55, 445 (1966).

43. Rüde, E., Mozes, E. and Sela, M. Biochem. 7, 2971 (1968).

44. Mozes, E. and Sela, M. Israel J. Med. Sci. 5, 267 (1969).

45. Benacerraf, B., Nussenzweig, V., Maurer, P. and Stylos, W. Israel J. Med. Sci. 5, 171 (1969).

46. Maurer, P.H. Ann. N.Y. Acad. Sci. 103, 549 (1963).

47. Gill, T.J., Gouls, H.J. and Kuntz, H.W. J. Biol. Chem. 239, 3083 (1964).

48. Sela, M. Ann. N.Y. Acad. Sci. 169, 23 (1970).

49. Richards, F.F., Sloane, R.W. and Haber, E. Biochem. 6, 476 (1967).

50. Levin, H.A., Levine, H. and Schlossman, S.F. J. Immunol. 104, 1377 (1970).

51. Kabat, E.A. J. Immunol. 97, 1 (1966).

52. Zolla, S. and Goodman, J.W. Immunochem. 4, 135 (1962).

53. Morgan, W.T.J. Ann. N.Y. Acad. Sci. 169, 118 (1970).

54. Cebra, J.J. J. Immunol. 86, 205 (1961).

55. Lapresle, C. and Webb, T. Bull. Soc. Chim. Biol. 46, 1701 (1964).

56. Lapresle, C. and Webb, T. Ann. Inst. Pasteur 99, 523 (1960).

57. Press, E.M. and Porter, R.R. Biochem. J. 83, 172 (1962).

58. Anderer, F.A. Biochim. Biophys. Acta 71, 246 (1963).

59. Benjamini, E., Young, J.D., Peterson, W.S., Leung, C.Y. and Shimizu, M. Biochem. 4, 2081 (1965).

60. Raynaud, M. Antibodies to Biologically Active Molecules, Vol. 1, (B. Cinader, ed.), Pergamon Press, Oxford, England.

61. Nussenzweig, V. and Seligmann, M. Rev. Hematol. 15, 451 (1960).

62. Metzger, H., Sharp, G. and Edelhoch, H. Biochem. 1, 205 (1962).

63. Mills, J.A. and Haber, E. J. Immunol. 91, 536 (1963).

64. Brown, R.K. J. Biol. Chem. 237, 1162 (1962).

65. Shinka, S., Imanishi, M., Kuwahara, O., Fujio, H. and Amano, T. Biken J. 5, 181 (1962).

66. Arnon, R. and Sela, M. Proc. Nat. Acad. Sci. U.S.A. 62, 163 (1969).

67. Omenu, G.S., Ontjes, D.A. and Anfinsen, C.B. Biochem. 9, 313 (1970).

68. Crumpton, M.J. and Wilkinson, J.M. Biochem. J. 94, 545 (1965).

69. Smyth, D.G., Stein, W.H. and Moore, S. J. Biol. Chem. 238, 227 (1963).

70. Anfinsen, C.B. Proc. Nat. Acad. Sci. U.S.A. 47, 1230 (1961).

71. Canfield, R.E. and Liu, A.K. J. Biol. Chem. 240, 1997 (1965).

72. Cusumano, C., Taniuchi, H. and Anfinsen, C.B. J. Biol. Chem. 243, 4769 (1968).

73. Edmundson, A.B. Nature 205, 883 (1965).

74. Kartha, G., Bello, J. and Harker, D. Nature 213, 862 (1967).

75. Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. Nature 206, 757 (1965).

76. Kendrew, J.C. Brookhaven Symposia in Biol. 15, 216 (1963).

77. Kendrew, J.C. Science 139, 1259 (1963).

78. Kendrew, J.C., Bodo, G., Dintzis, H.M., Parrish, R.G. and Wycoff, H. Nature 181, 662 (1958).

79. Kendrew, J.C., Dickerson, R.E., Strandberg, B.E., Hort, R.G., Davies, D.R., Phillips, D.C. and Shore, V.C. Nature 185, 422 (1960).

80. Kendrew, J.C., Watson, H.C., Strandberg, B.E., Dickerson, R.E., Phillips, D.C. and Shore, V.C. Nature 190, 666 (1961).

81. Benjamini, E., Young, J.D., Shimizu, M. and Leung, C.Y. Biochem. 3, 1115 (1964).

82. Stewart, J.M., Young, J.D., Benjamini, E., Shimizu, M. and Leung, C.Y. Biochem. 5, 3396 (1966).

83. Benjamini, E., Shimizu, M., Young, J.D. and Leung, C.Y. Biochem. 8, 2242 (1969).

84. Metzger, H., Wofsy, L. and Singer, S.J. Arch. Biochem. Biophys. 103, 206 (1963).

85. Singer, S.J. and Doolittle, R.F. Science 153, 13 (1966).

86. Shinka, S., Imanishi, M., Mujagawa, N., Inouye, M. and Tsugita, A. Biken J. 10, 89 (1967).

87. Fujio, H., Imanishi, M., Nishioka, K. and Amano, T. Biken J. 11, 207 (1968).

88. Fujio, H., Imanishi, M., Nishioka, K. and Amano, T. Biken J. 11, 219 (1968).

89. Arnon, R. Eur. J. Biochem. 5, 583 (1968).

90. Omenn, G.S., Ontjes, D.A. and Anfinsen, C.B. Biochem. 9, 304 (1970).

91. Brown, R.K., Delaney, R., Levine, L. and Van Vunakis, H. J. Biol. Chem. 234, 2043 (1959).

92. Maron, E., Shiozawa, C., Arnon, R. and Sela, M. Biochem. 10, 763 (1971).

93. Ovary, Z. Immunochem. 1, 241 (1964).

94. Sela, M. Naturwissenschaften 56, 206 (1969).

95. Reichlin, M., Hay, M. and Levine, L. Biochem. 2, 971 (1963).

96. Atassi, M.Z. Biochem. J. 103, 29 (1967).

97. Andres, S.F. and Atassi, M.Z. Biochem. 9, 2268 (1970)

98. Sela, M.,Schechter, B., Schechter, I. and Borek, F. Cold Spring Harbor Symp. Quant. Biol. 32, 537 (1967).

99. Sage, H.J., Deutsch, G.F., Fasman, G.D. and Levine, L. Immunochem. 1, 133 (1964).

100. Van Vunakis, H., Kaplan, J., Lehrer, H. and Levine, L. Immunochem. 3, 393 (1966).

101. Arnon, R., Sela, M., Yaron, A. and Sober, H. Biochem. 4, 948 (1965).

102. Schechter, I., Schechter, B. and Sela, M. Biochim. Biophys. Acta 127, 438 (1966).

103. Sela, M. Proc. Roy. Soc. B. 166, 188 (1966).

104. Schechter, B., Schechter, I. and Sela, M. J. Biol. Chem. 245, 1438 (1970).

105. Haimovich, J., Schechter, I. and Sela, M. Eur. J. Biochem. 7, 537 (1969).

106. Schlossman, S.F. and Levine, H. J. Immunol. 98, 211 (1967).

107. Kabat, E.A. Structural Concepts in Immunology and Immunochemistry, Holt, Rinehart and Winston, Inc., New York (1968).

108. Schlossman, S.F., Levine, H. and Yaron, A. Biochem. 7, 1 (1968).

109. Edmundson, A.B. and Hirs, C.H.W. J. Mol. Biol. 5, 663 (1962).

110. Lemberg, R. and Legge, J.W. Hematin Compounds and Bile Pigments, Interscience Publishers, New York (1949).

111. Antonini, E. Physiol. Rev. 45, 123 (1965).

112. Eylar, E.H., Banaszak, L.J. and Gurd, F.R.N. Abst. Amer. Chem. Soc. Meeting, Wash. D.C. 38c (March 1962).

113. Banaszak, L.J. and Gurd, F.R.N. J. Biol. Chem. 239, 1836 (1964).

114. Breslow, E., Beychok, S., Hardman, K.D. and Gurd, F.R.N. J. Biol. Chem. 240, 304 (1965).

115. Harrison, S.C. and Blout, E.R. J. Biol. Chem. 240, 299 (1965).

116. Crumpton, M.J. and Polson, A. J. Mol. Biol. 11, 722 (1965).

117. Atassi, M.Z. and Saplin, B.J. Biochem. 7, 688 (1968).

118. Atassi, M.Z. Nature 209, 1209 (1966).

119. Atassi, M.Z. and Caruso, D.R. Biochem. 7, 699 (1968).

120. Atassi, M.Z. Arch. Biochem. Biophys. 120, 56 (1967).

121. Atassi, M.Z. and Thomas, A.V. Biochem. 8, 3385 (1969).

122. Atassi, M.Z. Biochem. J. 102, 478 (1967).

123. Crumpton, M.J. and Small, P.A. J. Mol. Biol. 26, 143 (1967).

124. Epand, R.M. and Scheraga, H.A. Biochem. 7, 2864 (1968).

125. Singhal, R.P. and Atassi, M.Z. Biochem. 9, 4252 (1970).

126. Atassi, M.Z. and Singhal, R.P. Biochem. 9, 3854 (1970).

127. Atassi, M.Z. Biochem. 7, 3078 (1968).

128. Crumpton, M.J. Nature 215, 17 (1967).

129. Givas, J., Centeno, E.R., Manning, M. and Sehon, A.H. Immunochem. 5, 314 (1968).

130. Crumpton, M.J., Law, H.D. and Strong, R.C. Biochem. J. 116, 923 (1970).

131. Goodman, J.W. Immunochem. 6, 139 (1969).

132. Banaszak, L.J., Eylar, E.H. and Gurd, F.R.N. J. Biol. Chem. 238, 1989 (1963).

133. Breslow, E. J. Biol. Chem. 239, 486 (1964).

134. Beychok, S. and Blout, E.R. J. Mol. Biol. 3, 769 (1961).

135. Urnes, P.J., Imahori, K. and Doty, P. Proc. Nat. Acad. Sci. U.S.A. 47, 1635 (1961).

136. Ishizaka, K., Ishizaka, T. and Hornbrook, M.M. J. Immunol. 98, 490 (1967).

137. Edelman, G.M. and Gall, W.E. Ann. Rev. Biochem. 38, 415 (1969).

138. Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishouser, U. and Waxdal, M.J. Proc. Nat. Acad. Sci. U.S.A. 63, 78 (1969).

139. Porter, R.R. Basic Problems in Neoplastic Disease (A. Gellhorn and E. Hirschberg, eds.), Columbia University Press, New York (1962).

140. Pain, R.H. Biochem. J. 88, 234 (1963).

141. Small, P.A. Kehn, J.E. and Lamm, M.E. Science 142, 393 (1963).

142. Porter, R.R. Biochem. J. 73, 119 (1959).

143. Edelman, G.M. and Poulik, M.D. J. Exp. Med. 113, 861 (1961).

144. Nisonoff, A., Wissler, F.C., Lipman, L.N. and Woernley, D.L., Arch. Biochem. Biophys. 89, 230 (1960).

145. Nisonoff, A., Markus, G. and Wissler, F.C. Nature 189, 293 (1961).

146. Cahnmann, H.J., Arnon, R. and Sela, M. J. Biol. Chem. 241, 3247 (1966).

147. Palmer, J.L. and Nisonoff, A. Biochem. 3, 863 (1964).

148. Hong, R. and Nisonoff, A. J. Biol. Chem. 240, 3883 (1965).

149. Putnam, F.W. Science 163, 633 (1969).

150. Press, E.M. Givol, D., Piggot, P.J., Porter, R.R. and Wilkinson, J.M. Proc. Roy. Soc. B 166, 150 (1966).

151. Hill, R.L., Delaney, R., Lebovitz, H.E. and Fellows, R.E. Proc. Roy. Soc. B. 166, 159 (1966).

152. Miller, F. and Metzger, H. J. Biol. Chem. 240, 4740 (1965).

153. Mihaesco, C. and Seligmann, M. Immunochem. 5, 457 (1968).

154. Miller, F. and Metzger, H. J. Biol. Chem. 241, 1732 (1966).

155. Cohen, S. and Porter, R.R. Adv. Immunol. 4, 287 (1964).

156. Haurowitz, F. Physiol. Rev. 45, 1 (1965).

157. Fleischman, J.B. Ann. Rev. Biochem. 35, 835 (1966).

158. Hoglund, S. and Levin, O. J. Mol. Biol. 12, 866 (1965).

159. Valentine, R.C. and Green, N.M. J. Mol. Biol. 27, 615 (1967).

160. Feinstein, A. and Rowe, A.J. Nature 205, 147 (1965).

161. Green, N.M. Adv. Immunol. 11, 1 (1969).

162. Noelken, M.E., Nelson, C.A., Buckley, C.E. and Tanford, C.E. J. Biol. Chem. 240, 218 (1965).

163. Jirgensons, B. Arch. Biochem. Biophys. 74, 57 (1958).

164. Winkler, M. and Doty, P. Biochim. Biophys. Acta 54, 448 (1961).

165. Edelman, G.M., Olins, D.E., Gally, J.A. and Zinder, N.D. Proc. Nat. Acad. Sci. U.S.A. 50, 753 (1963).

166. Fougereau, M., Olins, D.E. and Edelman, G.M. J. Exp. Med. 120, 349 (1964).

167. Metzger, H. and Mannik, M. J. Exp. Med. 120, 765 (1964).

168. Olins, D.E. and Edelman, G.M. J. Exp. Med. 119, 789 (1964).

169. Roholt, O., Onoue, K. and Pressman, D. Proc. Nat. Acad. Sci. U.S.A. 51, 173 (1964).

170. Fleischman, J.B., Porter, R.R. and Press, E.M. Biochem. J. 88, 220 (1963).

171. Utsumi, S. and Karush, F. Biochem. 3, 1329 (1964).

172. Haber, E. and Richards, F.F. Proc. Roy. Soc. B. 166, 176 (1966).

173. Kelly, K. Ph.D. Thesis McGill University, Montreal, Canada (1970).

174. Pressman, D. and Roholt, O. Proc. Nat. Acad. Sci. U.S.A. 47, 1606 (1961).

175. Roholt, O.A., Radzimski, G. and Pressman, D. Science 141, 726 (1963).

176. Metzger, H., Wofsy, L. and Singer, S.J. Biochem. 2, 979 (1963).

177. Metzger, H., Wofsy, L. and Singer, S.J. Proc. Nat. Acad. Sci. U.S.A. 51, 612 (1964).

178. Good, A.H., Ovary, Z. and Singer, S.J. Biochem. 7, 1304 (1968).

179. Choules, G.L. and Singer, S.J. Immunochem. 3, 21 (1966).

180. Weinstein, Y., Wilchek, M. and Givol, D. Biochem. Biophys. Res. Comm. 35, 694 (1969).

181. Singer, S.J. and Thorpe, N.O. Proc. Nat. Acad. Sci. U.S.A. 60, 1371 (1968).

182. Roholt, O.A., Radzimski, G. and Pressman, D. J. Exp. Med. 125, 191 (1967).

183. Cathou, R. and Haber, E. Biochem. 6, 513 (1967).

184. Koshland, M.E., Reisfeld, R.A. and Dray, S. Immunochem. 5, 471 (1968).

185. Doolittle, R.F. and Singer, S.J. Proc. Nat. Acad. Sci. U.S.A. 54, 1773 (1965).

186. Parker, C.W., Yoo, T.J., Johnson, M.C. and Godt, S.M. Biochem. 6, 3408 (1967).

187. Spragg, J., Schröder, E., Stewart, J.M., Austen, K.F. and Haber, E. Biochem. 6, 3933 (1967).

188. Hsia, J.C. and Piette, L.H. Arch. Biochem. Biophys. 129, 296 (1969).

189. Oudin, J. Compt. Rend. 242, 2606 (1956).

190. Kelus, A.S. and Gell, P.G.H. J. Exp. Med. 127, 215 (1968).

191. Nisonoff, A., MacDonald, A.B., Hopper, J.E. and Daugharty, H. Fed. Proc. 29, 72 (1970).

192. Haber, E. Ann. Rev. Biochem. 37, 497 (1968).

193. Karush, F. J. Amer. Chem. Soc. 71, 363 (1949).

194. Nisonoff, A. and Pressman, D. J. Immunol. 80, 417 (1958).

195. Little, J.R. and Eisen, H.N. Biochem. 5, 3385 (1966).

196. Eisen, H.N. and Siskind, G.W. Biochem. 3, 996 (1964).

197. McGuigan, J.E., Simms, E.S. and Eisen, H.N. Biochem. 7, 1929 (1968).

198. Koyama, J., Grossberg, A.L. and Pressman, D. Biochem. 7, 1935 (1968).

199. Brenneman, L. and Singer, S.J. Proc. Nat. Acad. Sci. U.S.A. 60, 258 (1968).

200. Eisen, H.N., Simms, E.S., Little, J.R. and Steiner, L.A. Fed. Proc. 25, 559 (1966).

201. Little, J.R. and Counts, R.B. Biochem. 8, 2729 (1969).

202. Haber, E. Fed. Proc. 29, 66 (1970).

203. Braun, D.G., Eichmann, K. and Krause, R.M. J. Exp. Med. 129, 809 (1969).

204. Nisonoff, A., Zappacosta, S. and Jueziz, R. Cold Spring Harbor Symp. Quant. Biol. 32, 89 (1967).

205. Eisen, H.N., Michaelides, M.C., Underdown, B.J., Schulenberg, E.P. and Simms, E.S. Fed. Proc. 29, 78 (1970).

206. Jaffe, B.M., Eisen, H.N., Simms, E.S. and Potter, M. J. Immunol. 103, 872 (1969).

207. Terry, W.D., Ashman, R.F. and Metzger, H. Immunochem. 7, 257 (1970).

208. Terry, W.D., Matthews, B.W. and Davies, D.R. Nature 220, 239 (1968).

209. Sehon, A.H. Brit. Med. Bull. 19, 183 (1963).

210. Weliky, N. and Weetall, H.H. Immunochem. 2, 293 (1965).

211. Gurvitch, A.E., Kapner, R.B. and Nezlin, R.S. Biokhimya 24, 144 (1959).

212. Kreiter, V.P. and Pressman, D. Immunochem. 1, 91 (1964).

213. Robbins, J.B., Haimovich, J. and Sela, M. Immunochem. 4, 11 (1967).

214. Landsteiner, K. and Van der Scheer, J. Exp. Med. 63, 325 (1936).

215. Isliker, H.C. Ann. N.Y. Acad. Sci. 57, 225 (1953).

216. Yagi, Y., Engel, K. and Pressman, D. J. Immunol. 85, 375 (1960).

217. Gyenes, L. and Sehon, A.H. Can. J. Biochem. Physiol. 38, 1249 (1960).

218. Sun, K. Ph.D. Thesis, McGill University, Montreal, Canada (1967).

219. Centeno, E.R. and Sehon, A.H. Fed. Proc. 25, 729 (1966).

220. Omenn, G.S., Ontjes, D.A. and Anfinsen, C.B. Nature 225, 189 (1970).

221. Manecke, G. Pure Appl. Chem. 4, 507 (1962).

222. Froese, A. and Sehon, A.H. Can. J. Biochem. Physiol. 39, 1067 (1961).

223. Fischer, E. and Fourneau, E. Ber. Deut. Chem. Ges. 34, 2868 (1901).

224. Curtius, T. J. Prakt. Chem. 70, 57 (1904).

225. Bergmann, M. and Zervas, L. Ber. 65, 1192 (1932).

226. Sifferd, R.H. and du Vignaud, V. J. Biol. Chem. 108, 753 (1935).

227. Harington, C.R. and Mead, T.H. Biochem. J. 29, 1602 (1935).

228. Wieland, T., Kern, W. and Sehring, R. Ann. Chem. 569, 117 (1950).

229. Boissonnas, R.A. Helv. Chim. Acta 34, 874 (1951).

230. Vaughan, J.R. J. Amer. Chem. Soc. 73, 3547 (1951).

231. Anderson, G.W., Welcher, A.D. and Young, R.W. J. Amer. Chem. Soc. 73, 501 (1951).

Leaf 113 omitted

in page numbering.

232. Du Vignaud, V., Ressler, C., Swan, J.M., Roberts, C.W., Katsoyannis, P.G. and Gordon, S. J. Amer. Chem. Soc. 75, 4879 (1953).

233. Merrifield, R.B. J. Amer. Chem. Soc. 86, 304 (1964).

234. Merrifield, R.B. J. Org. Chem. 29, 3100 (1964).

235. Merrifield, R.B. Biochem. 3, 1385 (1964).

236. Merrifield, R.B. Endeavour 24, 3 (1965).

237. Marshall, G.R. and Merrifield, R.B. Biochem. 4, 2394 (1965).

238. Merrifield, R.B. Science 150, 178 (1965).

239. Marglin, A. and Merrifield, R.B. J. Amer. Chem. Soc. 88, 5051 (1966).

240. Katsoyannis, P.G. Amer. J. Med. 40, 652 (1966).

241. Gutte, B. and Merrifield, R.B. J. Amer. Chem. Soc. 91, 501 (1969).

242. Hirschmann, R., Nutt, R.F., Veber, D.F., Vitali, R.A., Varga, S.L., Jacob, T.A., Holly, F.W., and Denkewalter, R.G. J. Amer. Chem. Soc. 91, 507 (1969).

243. Li, C.H. and Yamashiro, D. J. Amer. Chem. Soc. 92, 7608 (1970).

244. Merrifield, R.B. Rec. Prog. Hormone Res. 23, 451 (1967).

245. Merrifield, R.B. Adv. Enzymol. 32, 221 (1969).

246. Bodansky, M. and Ondetti, M.A. Peptide Synthesis, Interscience, New York (1966).

247. Greenstein, J.P. and Winitz, M. Chemistry of the Amino Acids, John Wiley and Sons, Inc., New York (1961).

248. Schroder, E. and Lubke, K. The Peptides, Academic Press, New York (1965).

249. Sheehan, F.C. and Hess, G.P. J. Amer. Chem. Soc. 77, 1067 (1955).

250. Bodansky, M. Nature 175, 685 (1955).

251. Guttman, S. and Boissonnas, R.A. Helv. Chim. Acta 42, 1257 (1959).

252. Lenard, J. and Robinson, A.B. J. Amer. Chem. Soc. 89, 181 (1967).

253. Vogel, A. Quantitative Inorganic Analysis, 3rd ed., John Wiley and Sons, Inc., New York (1961).

254. Baxter, J.W.M., Manning, M. and Sawyer, W.H. Biochem. 8, 3592 (1969).

255. Pictet, A. and Geleznoff, A. Ber. 36, 2219 (1903).

256. Stewart, J.M. and Young, J.D. Solid-Phase Peptide Synthesis, Freeman and Co., San Francisco, Calif. (1969).

257. Spackman, D.H., Stein, W.H. and Moore, S. Anal. Chem. 30, 1190 (1958).

258. Bayer, E., Eckstein, H., Hagele, K., Konig, W.A., Bruning, W., Hagenmaier, H. and Parr, W. J. Amer. Chem. Soc. 92, 1735 (1970).

259. Hagenmaier, H. Tetrahedron Letters no. 4, 283 (1970).

260. Takashima, H., Merrifield, R.B. and du Vignaud, V. J. Amer. Chem. Soc. 90, 1323 (1968).

261. Manning, M. J. Amer. Chem. Soc. 90, 1348 (1968).

262. Gish, D.T., Katsoyannis, P.G., Hess, G.P. and Stedman, R.J. J. Amer. Chem. Soc. 78, 5954 (1956).

263. Paul, R. and Kende, A.S. J. Amer. Chem. Soc. 86, 741 (1964).

264. Chillemi, F. and Merrifield, R.B. Biochem. 8, 4344 (1969).

265. Shaltiel, S. Biochem. Biophys. Res. Comm. 29, 178 (1967).

266. Velick, S.F., Parker, C.W. and Eisen, H.N. Proc. Nat. Acad. Sci. U.S.A. 46, 1470 (1960).

267. Saha, A., Karush, F. and Marks, R. Immunochem. 3, 279 (1966).

268. Hercules, D.M. Fluorescence and Phosphorescence Analysis, Interscience Publishers, New York (1966).

269. Velick, S.F. Light and Life, (W.D. McElroy and B. Glass, eds.), Johns Hopkins Press, Baltimore, Md. (1961).

270. Little, J.R. and Eisen, H.N. Biochem. 6, 3119 (1967).

271. Little, J.R. and Eisen, H.N. J. Exp. Med. 129, 247 (1969).

272. Konev, S.V. Fluorescence and Phosphorescence of Proteins and Nucleic Acids, Plenum Press, New York (1967).

273. Seliger, H.H. and McElroy, W.D. Light; Physical and Biological Action, Academic Press, New York (1965).

274. Hardman, K.D., Eylar, E.H., Ray, D.K., Banaszak, L.J. and Gurd, F.R.N. J. Biol. Chem. 241, 432 (1966).

275. Ornstein, L. and Davis, B.J. Disc Electrophoresis, preprinted by Distillation Products Industries (Eastman Kodak Co.), Rochester, N.Y.

276. Yagi, Y., Maier, P., Pressman, D., Arbesman, C.E. and Reisman, R.E. J. Immunol. 91, 83 (1963).

277. Noble, R.W., Reichlin, M. and Gibson, Q.H. J. Biol. Chem. 244, 2403 (1969).

278. Luderitz, O., Staub, A.M. and Westphal, O. Bact. Rev. 30, 192 (1966).

279. Centeno, E.R., Givas, J., Ajdukovic, D., Manning, M. and Sehon, A.H. Internat. Symp. Macromol. Chem., IUPAC (1968).