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Antibody Active Sites and Immunoglobulin Molecules

Recent studies give more details of the structure and function of antibodies and pathological immunoglobulins.

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Antibody molecules show a remarkably high degree of specificity in their reaction, or reversible combination, with the particular kind of antigen molecule that elicited their production. Since the number of different antigens is enormous, the numbers of distinguishably different antibody molecules that an animal can produce must be correspondingly large. (A guess is that at least 10^5 kinds of antibodies can be generated in an animal, but the upper limit on this number is not really known.) In spite of this great diversity, however, antibodies all belong to a characteristic family of proteins, the immunoglobulins, and are chemically and physically closely related. One of the central questions in the study of immunological phenomena is how this combination of great diversity and great similarity of antibodies is achieved at the molecular level.

Antibody molecules contain localized regions, or active sites, which participate directly in the specific combination with the antigen. The antibody active sites are considered (*1*) to have a three-dimensional structure which fits in complementary fashion to the antigenic determinant. It was once thought (*2*) that different antibody molecules might have the same amino acid composition and sequence, but differ in the ways in which the active sites fold into a specific conformation (perhaps under the direct influence of the antigen). In recent years, however, differences in the compositions of the amino acids have been detected among the immunoglobulins, although whether these occur within the active-site regions and are directly related to antibody specificity has not been clear. In addition, recent studies of the reversibility of the molecular conformations of immunoglobulins have been interpreted to indicate that amino acid differences mediate antibody specificity. If the detailed relationship between chemical structure and antibody specificity were understood, it would no doubt shed light on a related mystery, namely, what are the unique biosynthetic mechanisms which generate this great multiplicity of related proteins?

The problem of antibody structure is complicated by several factors. One is that immunoglobulin molecules generally consist of two kinds of polypeptide chains, referred to as heavy and light (*3*, *4*). The roles of these chains in determining antibody structure and specificity have been the subject of much recent investigation and controversy. Second, it is clear that among heavy chains and among light chains there are amino acid differences which have nothing to do with antibody specificity, such as those on the heavy chains which are responsible for the different subclasses of the immunoglobulins (IgG, IgA, IgM, IgD; see *5*), and those on both heavy and light chains which are responsible for phenotypic differences among immunoglobulins. Third, all antibody preparations so far investigated, even those isolated in a pure state from individual animals immunized with apparently well-defined antigenic determinants (haptens), have exhibited a heterogeneity of electrophoretic mobilities and binding constants which suggests that they are chemically heterogeneous as well. For these reasons, structural studies such as peptide fingerprinting, which have been used so successfully with other protein systems, have not yielded significant results with antibodies (*6*).

In order to study the chemical structure of antibodies in detail, the complexities raised by these problems must be circumvented. One approach would be to focus attention directly on the active sites of the antibody molecules themselves. There are two such sites per antibody molecule in the most common subclass of immunoglobulins, IgG. These sites, comprising the amino acid residues that participate directly in the binding of the antigenic determinant, constitute only a small portion of the order of a few percent of the mass of the whole antibody molecule. We have concentrated for the last several years on developing and utilizing a method for studying the amino acid structure of these active sites. It seems appropriate at this stage to summarize the current status of this research and the conclusions and speculations derived from it.

Affinity Labeling

In the past decade, considerable information about the active sites of a variety of enzymes has been obtained by attaching chemical groups (labels) specifically to some amino acid residue in the active sites. Once a stable linkage is thus formed, the label serves to identify peptide fragments arising from

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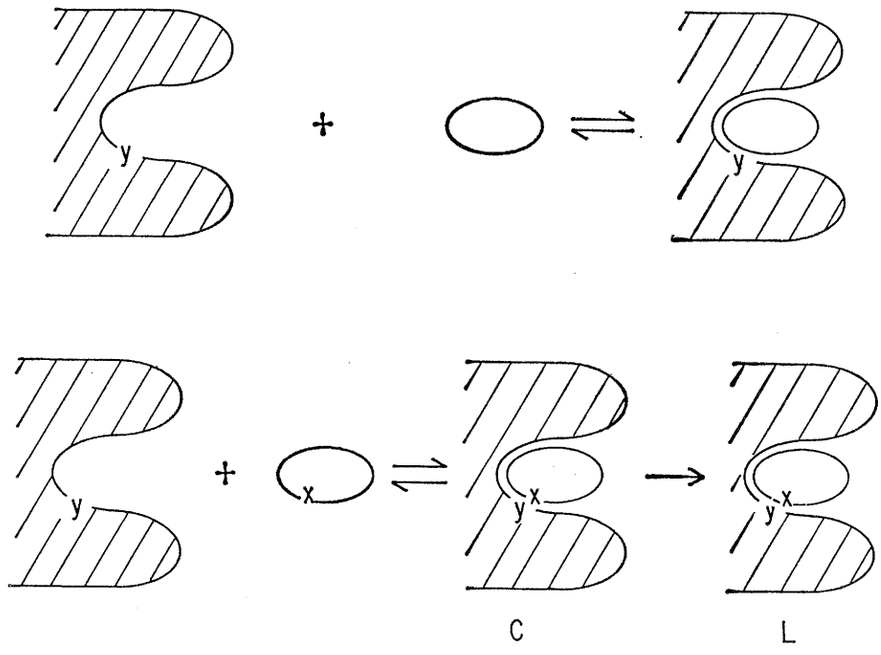


Fig. 1. The method of affinity labeling illustrated. At the top, the typical reversible combination of a hapten with its specific antibody site is represented. At the bottom, the hapten, modified by the attachment of the group x, first combines reversibly with the specific site to give a complex C; while in the site, the group x reacts to form a covalent bond with a suitable amino acid residue y in the site, yielding the labeled product L.

the site upon systematic degradation of the labeled enzyme. In labeling enzymes, advantage is usually taken of the fact that they have one or more uniquely reactive residues in their active sites which participate directly in the catalytic activity of the enzymes. The active sites of antibody molecules, however, would not be expected to contain such unique residues since they exert no catalytic activity. To label such sites, Leon Wofsy and one of us (Singer) 5 years ago hit upon a new method called affinity labeling (*7*). Similar studies with enzymes were independently conceived and carried out in a number of laboratories (*8*). In the method of affinity labeling, a specific labeling reagent for a given antibody is synthesized which (i) can combine specifically with the particular antibody active sites and (ii) possesses a small functional group (group x) which can react readily with one or more amino acid residues to form irreversible covalent bonds. The basis of the method is illustrated in Fig. 1. Because a reversible complex (C) is initially formed between the reagent and the active site, the concentration of the reagent in the site becomes much greater than in free solution; this greatly increases the rate of covalent bond formation by group x with a suitable residue y within the site, as compared with other similar residues outside the site. Thus a high degree of specificity of labeling of the active sites is achieved. The labeled product (L in Fig. 1) may then be subjected to any of a variety of chemical and enzymatic treatments (so long as they do not destroy the label) under conditions which usually cause the loss of the combining capacity of the active sites of unlabeled antibody molecules. The label then identifies fragments present in the original active sites and permits their isolation and analysis.

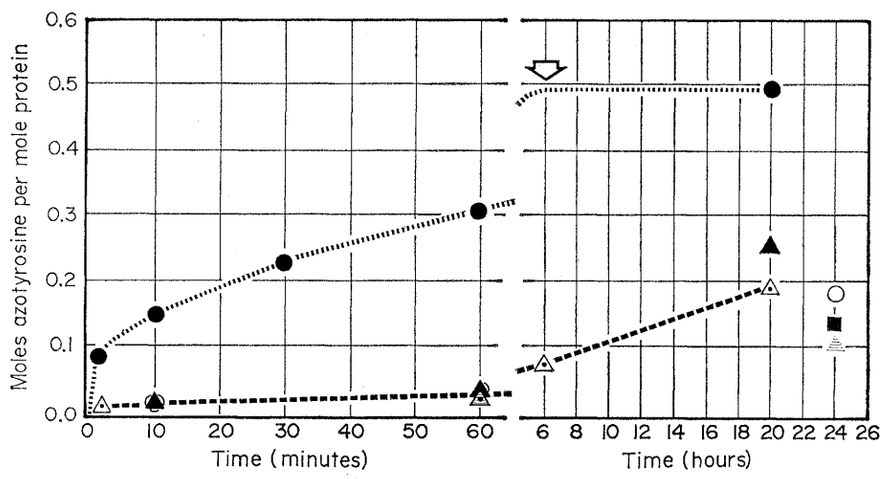


Fig. 2. Rate of formation of monoazotyrosine residues in the reaction of rabbit antibodies to *p*-azobenzenearsonate (R) and normal γ-globulin with *p*-(arsonic acid) benzenediazonium fluoborate (RDF) or *p*-(carboxylic acid) benzenediazonium fluoborate (CDF): ●, unprotected antibodies to R with RDF; ▲, protected antibodies to R with RDF; △, γ-globulin with RDF; ○, unprotected antibodies to R with CDF; ■, protected antibodies to R with CDF; shaded triangles, γ-globulin with CDF. The arrow indicates the fact, verified in separate experiments, that no further modification of unprotected antibodies to R with RDF is detected after 6 hours (*7*).

The method is best described by an example. Rabbit antibodies specific for the *p*-azobenzenearsonate (R) antigenic determinant, or hapten, have long been studied by immunochemists and can be isolated in the pure state. In our initial studies (*7*), the specific labeling reagent used with these antibodies was *p*-(arsonic acid) benzenediazonium fluoborate (RDF), in which the diazonium group is the functional group x in Fig. 1. The small size of the diazonium group insures that it falls well within the active site when RDF is reversibly bound to antibody to R. Diazonium compounds are well-known in protein chemistry and are capable of forming covalent azo bonds to tyrosine, histidine, lysine, and perhaps other amino acid residues (*9*). These derivatives have absorption spectra in the visible range, whereas the original diazonium salt does not. This permits the covalent bond formation between the reagent and antibody to be followed spectrophotometrically, and the azo bonds that are formed can be identified spectrally by comparison with simple model compounds. The results obtained are shown in part in Fig. 2.

1) As predicted by the elementary theory of affinity labeling, the rate of azo bond formation of RDF with anti-

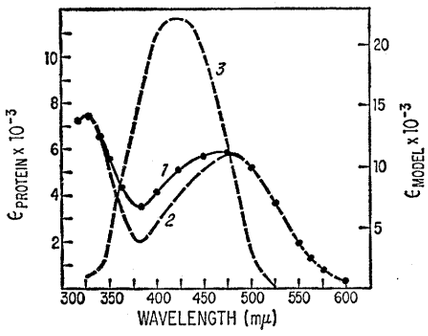


Fig. 3. Azo difference spectra of: 1, RDF-labeled antibodies to R (left coordinate); and spectra of the model compounds (right coordinate); 2, R-azo (*N*-chloracetyl) tyrosine; and 3, R-azo (*N*-acetyl) histidine, all in 0.15*M* NaOH (*13*). ϵ, Extinction coefficient.

bodies to R is markedly enhanced over that with nonantibody γ-globulin.

2) If the active sites of antibodies to R are first saturated ("protected") with an excess of the specific hapten *p*-nitrobenzenearsonate, the enhanced reaction with RDF is eliminated.

3) If, instead of RDF, the labeling reagent *p*-(carboxylic acid) benzenediazonium fluoborate (CDF) is used with antibodies to R, the reaction is not enhanced. This result was predicted, for although the two reagents are very similar and are equally reactive as diazonium compounds, CDF binds much more weakly than RDF to the active sites of antibodies to R.

4) Furthermore, the specific reaction of RDF with antibodies to R produces exclusively azo-tyrosine bonds, according to spectral analysis (Fig. 3), whereas the reaction of RDF with normal γ-globulin produces azohistidine and other linkages, as well as azotyrosine.

These results taken together provide strong evidence that one or more tyrosine residues in the active sites of antibodies to R form specific azo-derivatives with RDF.

Affinity labeling studies with three anti-hapten antibody systems of different specificities have been reported (Table 1). These include, in addition to antibodies to R, antibodies to 2,4 dinitrophenyl (DNP), and to *p*-azotrimethylphenylammonium (TMA). These haptens at physiological *p*H are negatively charged, neutral, and positively charged, respectively. With antibodies to DNP, the labeling reagents *p*-nitrobenzenediazonium fluoborate (PNBDF) and the *m*-isomer (MNBDF) have been extensively studied (*10*, *11*), and with antibodies to TMA, the reagent *p*-(trimethylammonium) benzenediazonium fluoborate (TDF) has been employed (*12*). In all cases the results have been remarkably similar. The labeling reagents reacted with their specific antibodies at greatly enhanced rates compared to nonspecific γ-globulin; the enhanced rates were eliminated in the presence of specific protectors of the antibody active sites; azotyrosine bonds were formed exclusively.

The evidence already presented makes it very likely that the affinity labels are indeed associated with the active sites of the antibodies. Further proof of this is the finding that, in the labeling of antibodies to DNP with the reagents PNBDF (*10*) and MNBDF (*11*), a loss of reversible binding sites occurs to an extent equivalent to the amount of label irreversibly bound (see Table 2 below). In other words, the affinity label irreversibly inactivates the antibody active site.

Affinity Labels on Heavy and Light Chains

While we were pursuing our initial studies of affinity labeling, the polypeptide chain structure of the immunoglobulins was worked out (*3*, *4*). The IgG molecule, for example, contains two heavy chains (molecular weight about 50,000) and two light chains (molecular weight about 25,000) linked together by disulfide bonds and noncovalent interactions. To determine how the affinity labels were distributed between the chains, we reduced the disulfide links between the chains and separated the chains from one another by the method of Fleischman *et al.* (*4*). To our surprise, we found the labels attached both to heavy and light chains (*13*); this has now been shown in all cases which we have studied so far (*11*, *12*).

Because this result was unexpected, we carried out a number of experiments to determine whether some kind of chemical artifact could have produced this result, but no such artifact could be discovered (*13*). On the contrary, the evidence is strong that the labels on both chains are specific. Spectral analyses have demonstrated that they are exclusively azotyrosine-linked. Furthermore, in studies with anti-DNP antibodies labeled with tritiated MNBDF (*11*), it has been shown (Table 2) that the presence of the protector during the labeling reaction markedly and equally diminishes the extent of labeling of both chains. Therefore, more than 90 percent of the labels on both chains are specifically localized to the antibody active sites in this system.

It is especially noteworthy that with antibodies to all three haptens, the distribution of label between the heavy and light chains is almost constant (Table 1), approximately 2:1 on a molar basis. This ratio does not vary appreciably from one batch of antibody to another. Furthermore, over a range of ten-fold in the fraction of anti-DNP antibody sites labeled with ^{3}H-MNBDF (*11*), the ratio remains essentially unchanged. The significance of these facts is discussed later on.

Labeled Peptide Fragments

A central objective of these labeling studies has been the isolation and characterization of peptide fragments from the antibody active sites. Before embarking on such studies, however, it was necessary to prove that affinity labeling did indeed attach labels to the active sites and to develop labeling reagents of high specificity which could be detected with adequate sensitivity. Having accomplished these initial objectives with antibodies to DNP, using the tritiated labeling reagent MNBDF (*11*), we have begun enzyme degradation studies of the labeled heavy and light chains of this antibody preparation.

The initial studies (*14*) have largely involved peptide fragments produced by the action of trypsin, an enzyme which catalyzes the hydrolysis of arginyl and lysyl peptide bonds specifically. If a polypeptide preparation is homogeneous so that each molecule in the population has arginyl and lysyl residues in the same characteristic positions in the amino acid sequence, discrete and characteristic peptide fragments are produced by tryptic digestion. In view of the localization of radioactive label to tyrosine residues within the active-site regions of the two kinds of antibody chains, it would be expected that if these regions were identical from one molecule to the next in the population, the radioactivity should be localized to a homogeneous tryptic fragment from each chain. On the other hand, if the molecules in this region differed chemically, a heterogeneous distribution of

the radioactive label would then result.

The isolated, labeled chains of antibody to DNP were oxidized with performic acid (*15*) and digested with trypsin. The tryptic peptides were then examined by a number of methods, the most definitive of which is countercurrent distribution. The results of a 100-tube transfer experiment in the solvent system *n*-butanol:acetic acid:water are shown in Fig. 4. Several significant findings emerge.

1) The radioactivity is broadly and continuously distributed, and thus the labeled peptides from both chains appear to be heterogeneous. By contrast, most of the unlabeled tryptic peptides are discrete entities (*6*). In the experiment of Fig. 4, these peptides are largely concentrated in the aqueous phases of the first few tubes, and on paper electrophoresis and chromatography they yield discrete ninhydrin-positive spots. The heterogeneity of the labeled peptides cannot be attributed to artifacts of the preparative procedures (*14*), and must therefore reflect a true chemical heterogeneity immediately surrounding the labeled tyrosine residues of both chains in the original molecular population.

2) The radioactivity is much more favorably distributed into the organic phase than is the nitrogen of the tryptic peptides (Table 3). This indicates that the labeled peptides from both chains are predominantly hydrophobic in acidic media. This is an atypical property for peptide fragments; such unusual water-insoluble peptides are often referred to as "core" material. That such hydrophobicity characterizes the labeled fragments of *both* chains suggests that these particular segments of both chains are chemically similar.

3) Although the labeled peptides from both chains exhibit similar properties, they are distinguishable by their distribution. On the whole, the heavy-chain labeled peptides are more hydrophobic than those from the light chain. On paper chromatography in the same solvent system (not shown here), distributions of radioactivity similar to those seen in Fig. 4 are obtained.

In another set of experiments, the same tryptic digests were subjected to gel filtration to characterize the average sizes of the labeled fragments. The filtration medium was P-10 Biogel in the desorbing solvent (*16*) phenol: acetic acid:water (1:1:1); control experiments with model substances of known molecular weight (listed on the right side of Fig. 5) showed that the gel was indeed functioning as a molecular sieve under these conditions. With the heavy- and light-chain tryptic digests, the elution patterns of radioactivity (Fig. 5) indicate that the average sizes of the labeled tryptic peptides are about 25 amino acids for both the heavy and light chains. (On the other hand, the average size for all the tryptic peptides, as revealed by nitrogen analyses, is different for the two chains, as expected from the sum of their lysine and arginine contents.) From the

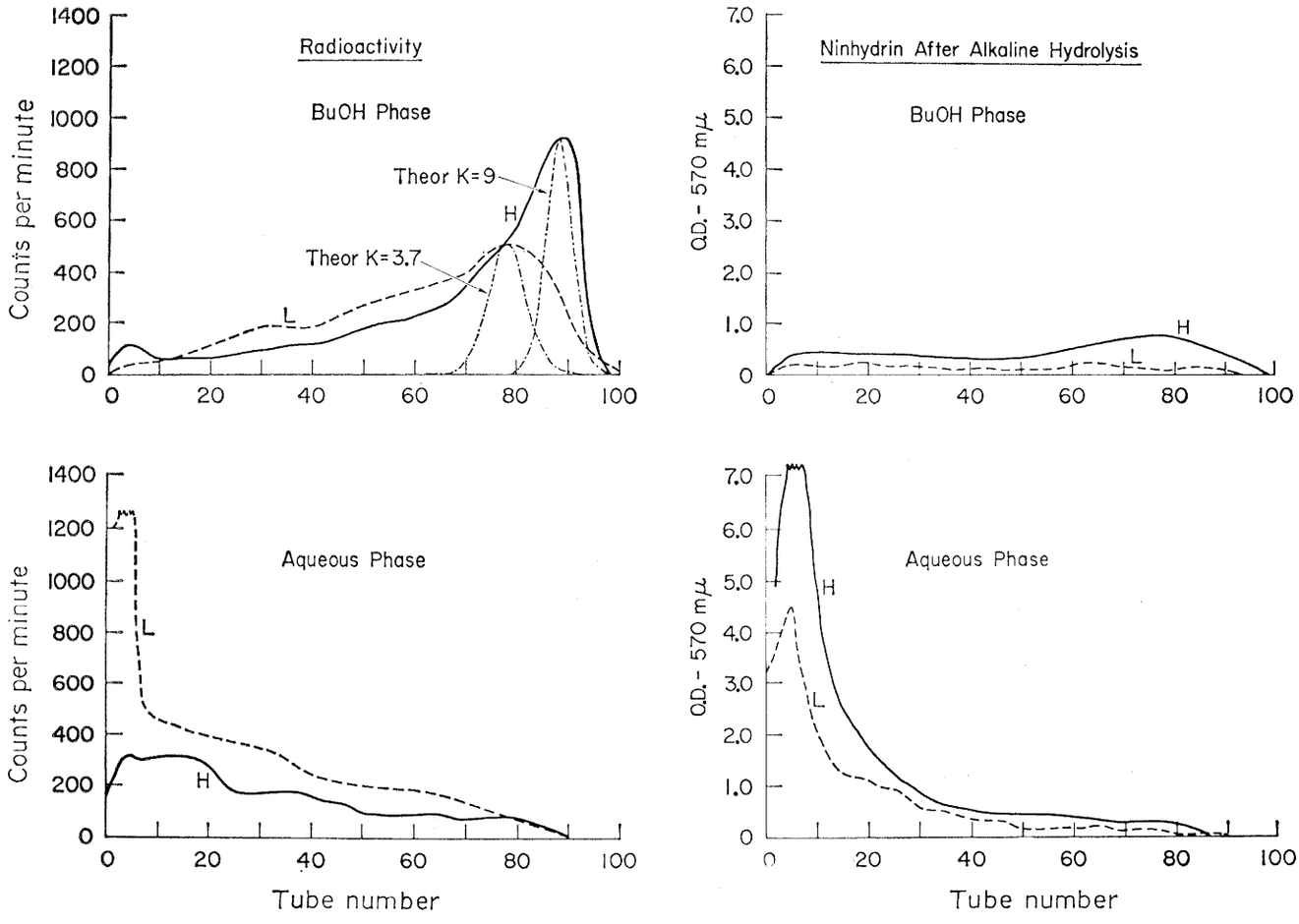


Fig. 4. Countercurrent distribution of tryptic digests of oxidized heavy (*H*) and light (*L*) chains of antibodies to 2,4 dinitrophenyl, labeled with tritiated *m*-nitrobenzenediazonium fluoborate. Solvent system, *n*-butanol (BuOH):acetic acid:water (4:1:5). Light-chain peptides subjected to 98 transfers, heavy-chain to 96. Upper and lower phase volumes, 12 and 10 ml, respectively. Temperature, 25°C. Theoretical curves are shown in upper left for two homogeneous substances with distribution coefficients, *K*, of 3.7 and 9 (*14*).

spreading of these patterns, it is clear that sizes are dispersed about this mean value, but there does not appear to be any significant amount of undigested material remaining after the trypsin treatment.

Peptide fragments have also been prepared from the oxidized labeled chains of antibodies to DNP by digestion with chymotrypsin. Preliminary analyses of these fragments by paper electrophoresis and chromatography (Fig. 6) reveal a remarkable similarity in the properties of the labeled chymotryptic fragments from heavy and light chains. Whereas unlabeled chymotryptic fragments of the two chains (as revealed by ninhydrin staining, not shown) are readily distinguished, the labeled fragments are indistinguishable. Very little of the label is released as the azoderivative of tryosine itself, so that the similarity between the digests of the two chains cannot be attributed to the presence of this compound in the free state. The indistinguishability of the labeled chymotryptic peptides from the two chains is highly significant in view of the fact that the labeled tryptic fragments of the two chains are readily distinguished by paper chromatography in the same solvent.

It is hoped that further studies of peptide fragments from affinity-labeled antibodies will provide more detailed chemical information about the labeled regions of both chains of a variety of antibodies. However, the data so far summarized in this article, in conjunction with results of other investigators, already led to a consistent picture of the structure of antibody sites, and of immunoglobulin molecules, which should be of value in guiding further experimental investigations. It is to these conclusions and speculations that we now turn.

Table 1. Affinity-labeling systems.

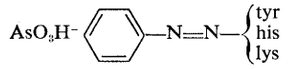
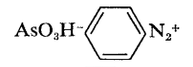
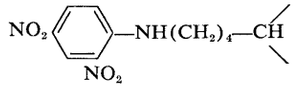
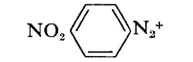
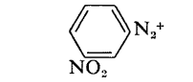
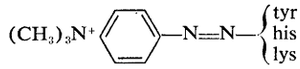
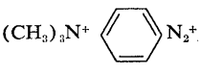
Antibody directed to		Labeling reagent	Mole ratio of label (heavy: light chain)	References
	(R)		2.1	*7, 13*
	(DNP)		1.3	*10, 13*
			1.8	*11*
	(TMA)		1.5	*12*

Table 2. Results obtained by labeling antibodies to 2,4 dinitrophenyl, with tritiated *m*-nitrobenzenediazonium fluoborate. Data are given in moles per mole of antibody. These experiments were terminated at a level of labeling of about 0.6 mole per mole of antibody in order to maintain a high specificity of labeling (see *11*).

Whole antibody				Heavy chains	Light chains	2 Heavy + 2 light
Reaction condition	Spectral, azotyrosine	^3H	Antibody binding sites lost	^3H	^3H	^3H
			Experiment 1			
Unprotected	0.58	0.68		0.215	0.117	0.66
Protected		.059		.013	.007	.04
			Experiment 2			
Unprotected	.61	.51	0.50±.05*	.136	.072	.42

* As measured by titration with 2-(2,4 dinitrophenylazo)-1-naphthol-3,6 disulfonic acid, disodium salt (*10*).

Specificity of Affinity Labeling

The observations, summarized above, which indicate that the affinity labels are attached within the active sites of the antibodies, satisfy all the operational criteria that have been employed in other cases of active-site labeling (*17*). It has occasionally been suggested that the tyrosine residues which become affinity labeled are not within, but only "close to," the active sites. This does not appear to us to be tenable. A specific labeling reagent is in a rather confined geometry within the site when it reacts to form a covalent bond (*7*). This is most dramatically demonstrated (*18*) by the fact that each of three different reagents which label the active site of chymotrypsin (Fig. 7) attaches to a different residue acknowledged to be within the site—histidine-57, methionine-192, or serine-195; this is so despite strong similarities in the nature and reactivities of the reagents. The labeling reagent is obviously not free to react at random with different residues in or "close to" the site.

Furthermore, an antibody active site generally encompasses a volume much larger than the diazonium labeling reagents used in our studies (*19*). In the case of antibody to DNP, for example, the evidence is strong (*20*) that the active site is complementary not only to the DNP group but also to the lysyl group to which the DNP is attached on the hapten-protein conjugate used as the immunizing antigen.

These considerations, therefore, strongly argue that the tyrosine residues labeled in our studies are within, and not simply "close to," the antibody active sites.

The presence of tyrosine in the active sites of several different anti-hapten antibodies, including antibody to R, has been inferred independently from the results of iodination studies (*21*), although there are some apparently conflicting results with antibody to TMA (*22*). Iodination inactivates these antibodies to a considerable extent, and a part of this inactivation is prevented by the presence of a specific protector of the active sites.

It is of considerable interest that tyrosine residues are present in the active sites of a fairly wide range of antibody specificities. On the other hand, since all the haptens we have studied are of benzenoid or closely related chemical structure, it is too early to generalize about the presence of tyrosine in all antibody active sites.

Structures of Active Sites

It is of great interest that the tyrosine residues which become affinity-labeled are found on both heavy and light chains of the antibody molecules. That both kinds of labels are equally specific to the sites is demonstrated by the data of Table 2, which show that protection of the active sites during the labeling reaction eliminates both labels.

What is particularly remarkable, however, is the fact that with all three

anti-hapten antibody systems so far studied and with different batches of antibody to DNP, with two different reagents and with two different amounts of label, the distribution of label between the heavy and light chains has always been nearly constant at 2:1 on a molar basis (Table 1). The significance of this result can best be understood by examining three possible explanations for the appearance of label on both chains.

1) In some active sites in an antibody population, the heavy chains may determine the specificity exclusively, whereas in others the light chains may do so. The ratio between the amounts of label found on the two kinds of chains then would depend on the ratio between the two kinds of antibody sites in the population.

2) Each active site in the population may contain a tyrosine residue from the heavy chain and another from the light, but the two tyrosines are differently situated in different sites. Because its geometry within the active site is relatively confined, the labeling reagent in a given site has a fixed probability of reacting with either the heavy- or the light-chain tyrosine. This probability would vary over a wide range, however, for different sites in the population. The ratio between the amounts of label found on the two kinds of chains, then, would depend on the distribution of active sites in the population.

3) The heavy- and light-chain tyrosines that become labeled may be present in all the active sites in an antibody population in nearly fixed positions and orientations relative to one another. The labeling reagent then would have a fixed probability of reacting with either the heavy- or the light-chain tyrosine in all the active sites.

It is clear that with either of the first two possibilities the ratio between the amounts of label found on the two chains might vary within wide limits. Only fortuitously would this ratio be 2:1; it could just as readily be 10:1 or 1:10 in different samples of the same antibody, depending on the more or less random distribution of active sites in the antibody population. Only the third possibility provides a reasonable explanation of the nonrandomness of the distribution of label.

The fact that the distribution of label on the heavy and light chains is 2:1 rather than 1:1 might be due to a greater fixed probability of reaction of the labeling reagent with a single heavy-chain tyrosine than with a single light-chain tyrosine in each site. This scheme would have an analogy in the finding (*23*) that iodoacetate reacts with both histidine-119 and histidine-12 in the active sites of bovine pancreatic ribonuclease, and in the constant ratio of 85:15. In any one site, however, one or the other histidine is modified. The 2:1 ratio might also be due, however, to the nearly equal probability of labeling of two different tyrosine residues on the heavy chains and one on the light chain in each active site. These two possibilities are difficult to distinguish experimentally at present. However, the close similarity of the labeled chymotryptic fragments of heavy and light chains of antibodies to DNP (Fig. 6) argues for the presence of a single type of labeled tyrosine on each chain; otherwise a second peak of labeled peptide material might have been expected in the chromatogram and electrophorogram of the heavy-chain digest.

Our view is, therefore, that a tyrosine residue on the heavy chain and another on the light chain come into close appo-

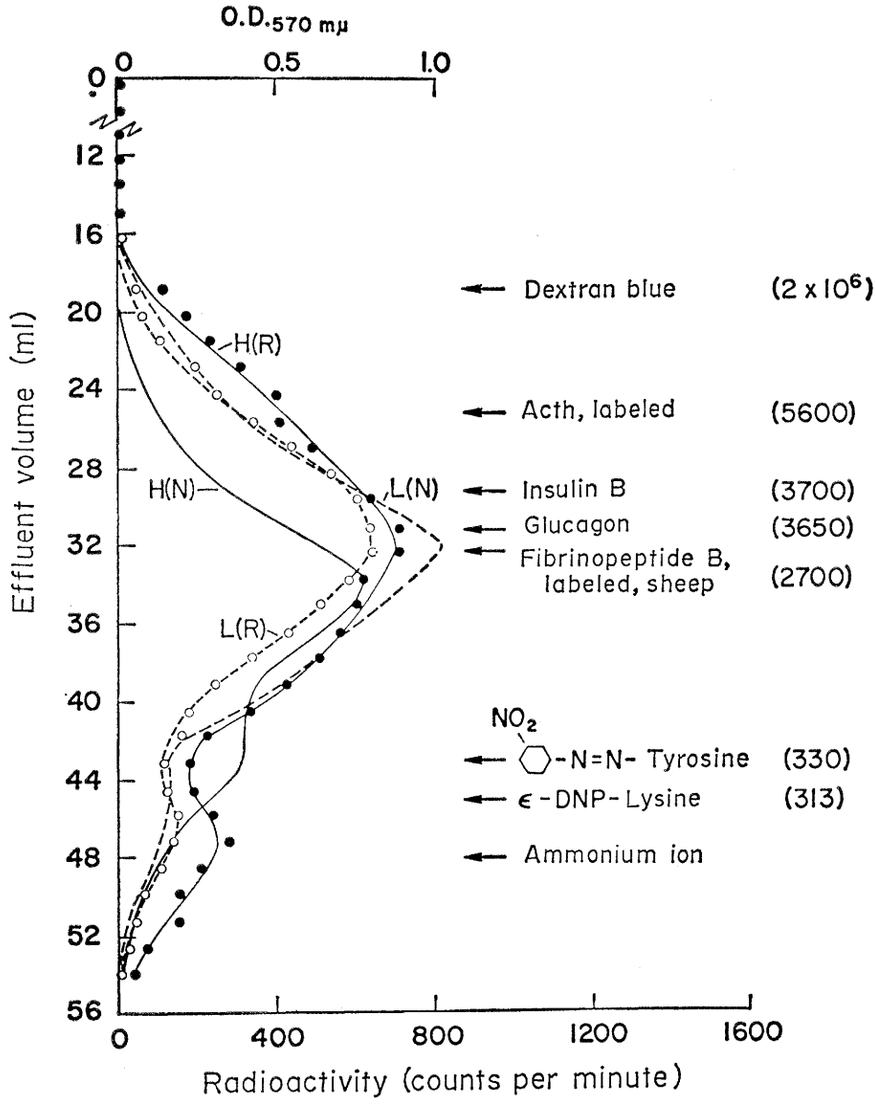


Fig. 5. Gel filtration of tryptic digests of oxidized heavy (*H*) and light (*L*) chains of antibodies to 2,4 dinitrophenyl (DNP) labeled with tritiated *m*-nitrobenzenediazonium fluoborate, and of various model substances, on Biogel P-10, 1.1 × 52-cm column, in phenol:acetic acid:water (1:1:1 by vol.). *H*(*R*) and *L*(*R*) refer to radioactivity distribution (lower coordinate) of *H* and *L* chain peptides, respectively; *H*(*N*) and *L*(*N*) to the corresponding nitrogen distribution, as measured by ninhydrin analyses after alkaline hydrolysis (upper coordinate). For the *H*(*R*) and *L*(*R*) curves, the data are included, but not for *H*(*N*) and *L*(*N*). Recovery of radioactivity from the column was 91 percent for heavy chains and 109 percent for light chains. Only the maxima of the elution profiles of the model compounds and polypeptides are shown. The molecular weights are given in parentheses, on the assumption that the labeled peptides are monoderivatized at all tyrosine, histidine, and free amino residues (*14*).

sition and are in nearly fixed relative positions and orientations in the active sites of the three anti-hapten antibodies which we have studied. This in turn suggests that immediately surrounding the tyrosines that become labeled on either chain there may be present a few amino acid residues which are the same or very similar in different antibody sites, so as to produce this strong chemical and conformational regularity among sites. We refer to this hypothetical region as a "conservative region."

Up to this point in our discussion, we have emphasized the chemical similarities and relationships among sites of different specificities. In view of these similarities, however, it is especially significant that the labeled tryptic peptides of both chains of antibodies to DNP (Fig. 4) are chemically heterogeneous. This heterogeneity should be viewed in terms of the well-known heterogeneity of anti-DNP antibodies in particular and all known anti-hapten antibodies in general. Although all the molecules in an anti-DNP antibody preparation are capable of combining with DNP-haptens, they bind these haptens in a nonuniform manner (*24, 20*) and exhibit a broad range of electrophoretic mobilities. Furthermore, the light chains of anti-DNP antibodies isolated from an individual, allotypically homozygous rabbit are grossly heterogeneous electrophoretically (*25*). Much the same properties characterize antibodies to other haptens. From the heterogeneity and the average sizes of the labeled tryptic peptides, we therefore conclude that, within about 25 amino acids sequentially removed from the labeled tyrosine residues in the active-site regions of both heavy and light chains of antibodies to DNP, substantial variations in composition and sequence occur from one chain to another in the antibody population. These variations within or sequentially adjacent to the antibody active sites must account, at least in part, for the heterogeneity of the antibody molecules.

If the active-site regions of antibody molecules of the same specificity can vary in amino acid composition and sequence, it appears highly probable that differences in antibody specificity are mediated by differences in amino acid structure. Comparative studies now in progress of affinity-labeled antibodies of different specificities may reveal such systematic chemical differences within different antibody active sites.

It has been inferred from other kinds of information that differences in antibody specificity are mediated by differences in amino acid structure. Koshland and Englberger (*26*) first reported small differences between the *total* amino acid compositions of purified anti-R and anti-TMA antibodies obtained from the same rabbit. It is not clear, however, that these differences are directly related to antibody specificity; in other instances, such differences apparently are not necessarily associated with the active sites (*27*). In addition, it has been shown (*28*) that, when the disulfide bridges in the antibody molecule and its chains are completely reduced in denaturing solvents and reoxidized in aqueous buffer, much of the specific activity is recovered. From these observations, Haber, and Whitney and Tanford, conclude that the primary structure determines the antibody specificity. This conclusion is based on the proposition that all conformational structure of the ac-

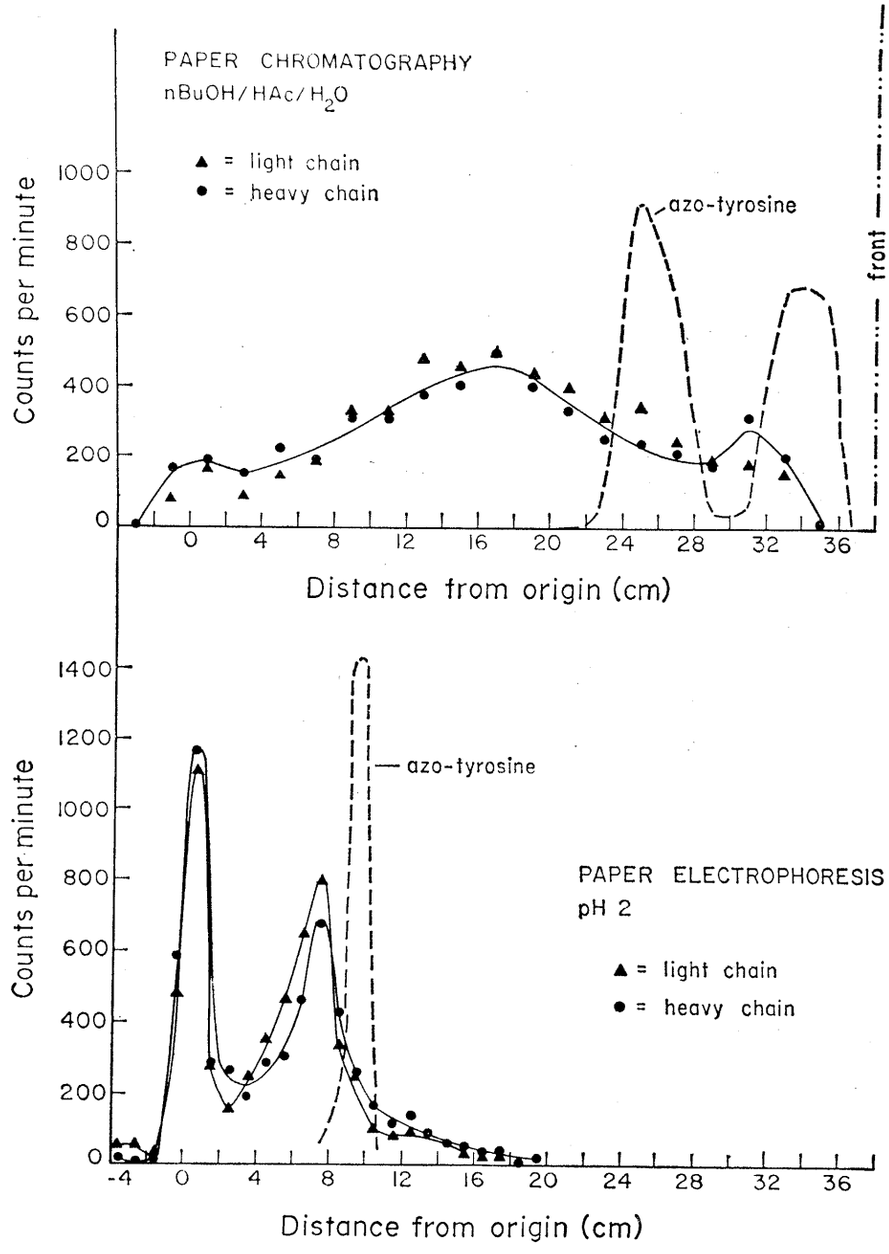


Fig. 6. Chymotryptic digests of heavy (●) and light (▲) chains of antibodies to 2,4 dinitrophenyl that have been labeled with tritiated *m*-nitrobenzenediazonium fluoborate and subjected (top) to paper chromatography in *n*-butanol:acetic acid:water, and (bottom) to paper electrophoresis at *p*H 2, and 3 kv. The distribution of the radioactivity of the digests is given. The distribution of the model compound *m*-nitrophenylazotyrosine under the same conditions, determined in parallel experiments, is also shown as dashed lines. (The reason for the second, faster-moving peak on chromatography of the model compound is not yet understood.)

Table 3. Countercurrent distribution of radioactivity and nitrogen of tryptic digests of chains from labeled antibody to 2,4 dinitrophenyl. Calculated from data shown in Fig. 4.

Tubes	Heavy chain		Light chain	
	Radioactivity (%)	Nitrogen* (%)	Radioactivity (%)	Nitrogen* (%)
0–49†	38.9	80.5	54.7	84.9
50–99‡	61.1	19.5	45.3	15.1

* Ninhydrin analyses after alkaline hydrolysis. † Distribution coefficients, K, equal to or less than 1.0. ‡ K values greater than 1.0.

tive sites is lost on complete reduction in denaturing media. It is difficult to be entirely certain of this, however, particularly if the active sites are unusually hydrophobic.

To summarize, we have concluded that antibody sites may contain a limited region (the conservative region) of considerable chemical similarity from one specificity to another, but that also closely associated with the active sites is a region which may vary in amino acid composition and sequence from one specificity to another. Although at first sight these two conclusions appear contradictory, they can be reconciled. If the postulate of a conservative region within antibody active sites is granted, there must be another region of the sites which is more importantly involved in discriminating the specific antigenic determinant. We assume that at least some of the chemical variability of the labeled tryptic peptides is located in this region, which we refer to as a "variable region." It would appear, therefore, that limited and similar regions of heavy and light chains may be utilized to construct antibody sites of different specificities and that different antibody sites are chemical perturbations of a particular confined region of the antibody molecule.

There is a striking analogy between the picture which thus emerges for antibody sites of a variety of specificities and that arising from the detailed studies that have been made of the amino acid structures in and adjacent to the active sites of the esterase enzymes; in particular, trypsin and chymotrypsin. As a result of a long series of investigations following the pioneering studies of Balls and Jansen (*29*), it is now universally accepted that a unique serine residue, one histidine residue (*30*), and probably another (*31*) are present in the active sites of both trypsin and chymotrypsin. It is remarkable that the critical serine residue occurs within a hexapeptide sequence that is identical for the two enzymes, and the two histidines in a nonadecapeptide sequence which is almost identical (*31*). Yet the two enzymes have quite different substrate specificities. In some manner, the active sites must be capable of selectively binding their respective substrates. It seems reasonable that portions of the hexapeptide and nonadecapeptide sequences just mentioned constitute a conservative region close to the amide bond of the bound specific substrate in both enzymes. Geometrically adjacent to this conservative region there should be a variable region which recognizes the specific substrate. This close analogy between different antibody and different enzyme active sites is schematically represented in Fig. 8.

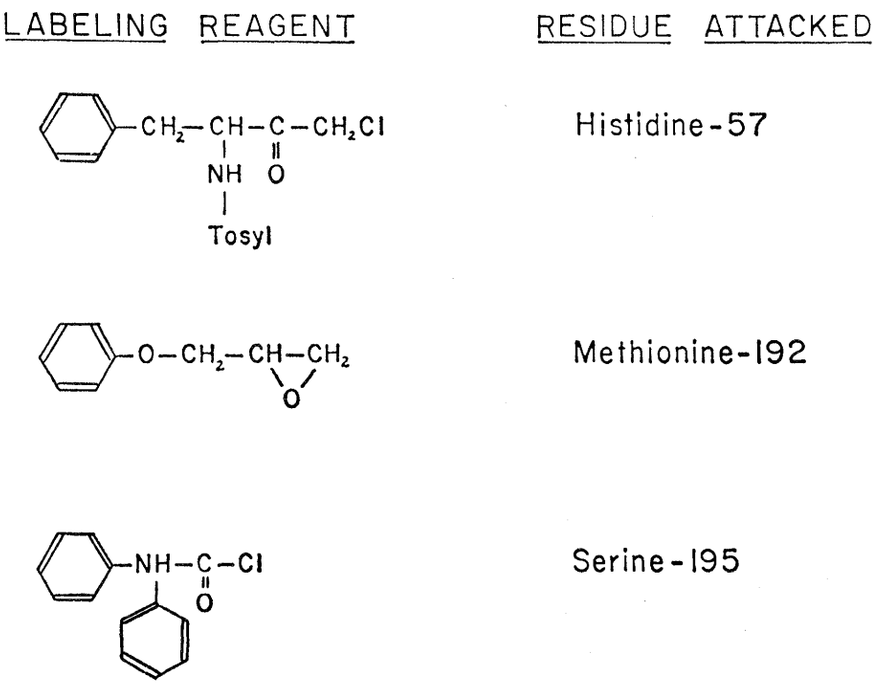


Fig. 7. Three different affinity-labeling reagents which label the active site of chymotrypsin. Each forms a covalent bond with a different amino acid residue in the site (*18*).

Another feature of our results which bears on the chemistry of antibody active sites is the unusual hydrophobicity revealed by a major portion of the labeled tryptic peptides from both the heavy and light chains of antibodies to DNP. Whereas only a small fraction of the total nitrogen of the mixture is extracted into the butanol phase, half or more of the radioactivity associated with each chain is transferred (Fig. 4 and Table 3). The properties of the labeled peptides from affinity-labeled antibodies to R and to TMA have not yet been adequately investigated. It is probably significant, however, that in qualitative studies of fragments of RDF-labeled anti-R antibodies (*32*) in which the label was followed visually by its azo color, labeled tryptic peptides from performate-oxidized heavy and light chains were found to remain predominantly at the origin on paper electrophoresis and to migrate rapidly on paper chromatography in butanol: acetic acid, exhibiting properties similar to those of the corresponding fragments of antibodies to DNP. Further studies of labeled peptide fragments from antibodies of different specificities should reveal more clearly whether such hydrophobic regions are more generally involved in antibody active sites. It is of interest in this connection that, for other reasons, it has been suggested that hydrophobic interactions play an important role in the formation of many antigen-antibody bonds (*33*).

Bence Jones Proteins

In connection with a discussion of the structure of antibody active sites, recent investigations of the properties of the Bence Jones proteins are of great

interest. These proteins appear in large quantities in the urine of humans suffering from multiple myeloma and in the urine of inbred mice in which myelomas are induced by chemical treatment (*34*). From any individual, the Bence Jones protein is often homogeneous. Whereas different Bence Jones proteins have many common properties, including some antigenic determinants, no two have been found to be entirely identical in physical and chemical properties. As a result of recent investigations (*35*) it is now widely accepted that a particular Bence Jones protein is a single kind of immunoglobulin light polypeptide chain. It appears likely that each homogeneous Bence Jones protein is one member of the very heterogeneous population of light chains found in the immunoglobulins of any normal individual. In other words, it is probable that a Bence Jones protein is not a defective light chain, but is rather a normal light chain proliferated because of a defective control of protein synthesis.

Examination of their tryptic peptides (*36*) has shown that different Bence Jones proteins of a given type share a considerable number of peptides in common, but that each contains some peptides that are unique to it. More recently, Hilschmann and Craig (*37*), by determining the partial amino acid sequence of two human type κ Bence Jones proteins differing widely in chemical composition, have found that of the more than 200 amino acids in the polypeptide chains, the 106 contiguous residues from the carboxyl ends are essentially invariant (region *i'* in Fig. 9); however, among the next more than 100 amino acids to the amino-terminal ends of the chains, substantial variations in composition and sequence occur between the two proteins (region *v'*, Fig. 9). This division of the chain into variant and essentially invariant halves has been confirmed with a third Bence Jones protein (*38*), and there is good evidence (*39*) that the structure applies generally to all normal light chains.

We may anticipate that complete amino acid sequences of a significant number of the Bence Jones proteins will reveal many important features of immunoglobulin structure and provide important clues about the mechanisms of antibody biosynthesis. Our purpose here, however, is to correlate the preliminary data for the amino acid sequences of the Bence Jones proteins with the results obtained from our studies of affinity-labeled antibodies.

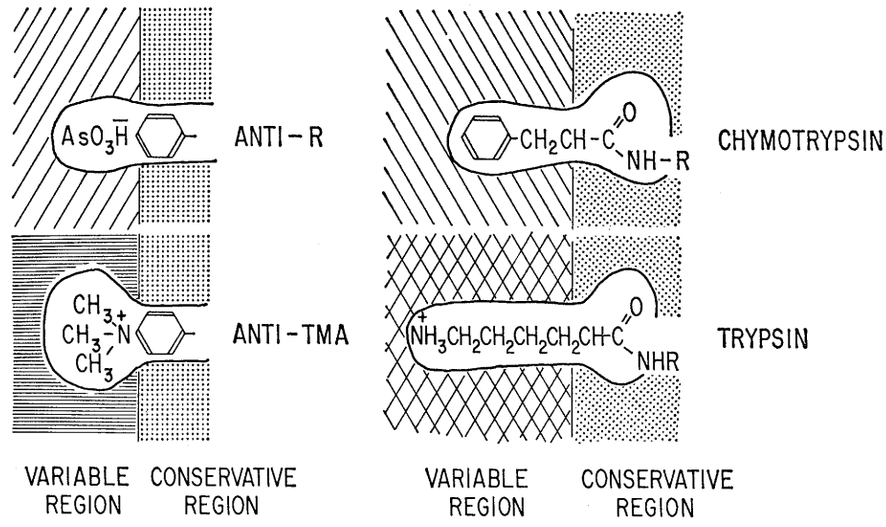


Fig. 8. Schematic representation of the three-dimensional structure of two antibody and the two enzyme active sites in which specific function is achieved by the juxtaposition of a region of variable composition and structure to a conservative region of essentially constant composition and structure.

Since the active-site regions of the light chains of affinity-labeled antibodies to DNP are chemically heterogeneous, we infer that these regions must be located in the variant amino-terminal halves of the light chains. Furthermore, in view of the hydrophobic properties and average size of about 25 residues of the affinity-labeled tryptic peptides, it is of interest that in one Bence Jones protein (Roy protein, *37*) there is within the variant half of the chain a hydrophobic tryptic peptide which contains one tyrosine residue among its 23 amino acids (residues 19 to 41 from the amino-terminal end). Although this tryptic peptide appears to vary in composition and sequence in a second Bence Jones protein (Cum protein, *37*), the nonapeptide sequence immediately surrounding the tyrosine residue, Leu-Asn-Try-Tyr-(Glu,Glu,Pro,Gly)-Lys (*38*) appears to be the same for all three Bence Jones proteins so far examined. In other words, within the variant half of the chain, there is this region of apparent invariance. Perhaps this invariant nonapeptide in part constitutes a portion of the conservative region of the antibody active sites (Fig. 8) postulated from the results of affinity-labeling studies.

In spite of the present limitations on such speculations, one may predict that further correlated sequence studies will permit unambiguous identification of the tyrosine residue on the light chain which becomes affinity-labeled within the antibody active sites.

As yet, no amino acid sequence studies of heavy chains have been reported that would complement those which have been initiated with the Bence Jones proteins. Myeloma cells proliferate distinct heavy chains as well as light chains, but the heavy chains do not appear to occur in the free state to any great extent, being covalently linked with the light chains in whole immunoglobulin molecules. As yet, little is known about the detailed structure of these heavy chains.

Relations of Heavy and Light Chains

Since the discovery of the two kinds of polypeptide chains in immunoglobulin molecules (*3, 4*), much interest has attached to their respective roles in the determination of the specificity of antibodies. This question is important not only to the function of antibodies but also to the problem of antibody biosynthesis because it defines, in part, the degree of complexity which the biosynthetic mechanisms must attain. At present, the published results of studies on the importance of heavy and light chains to antibody binding capacity and specificity are somewhat contradictory. It has frequently been observed that only the isolated heavy-chain fractions, and not the light-chain fractions, of antibodies retain specific binding capacity (*4, 40*); in two cases, the recovery of the original binding affinity of the antibodies in the isolated heavy-chain fraction alone has been very large (*41*). These results argue

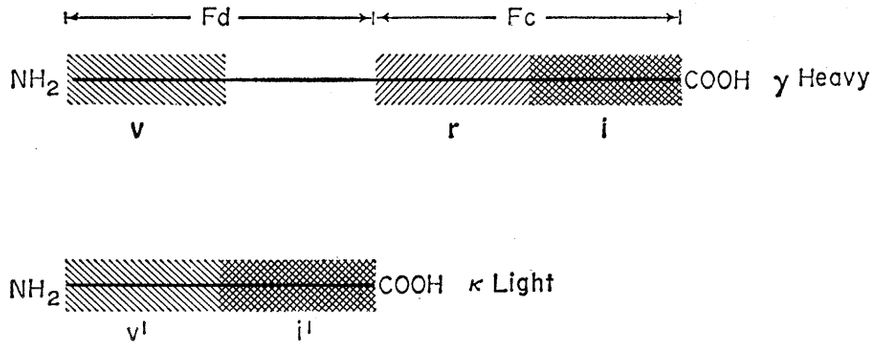


Fig. 9. A schematic representation of the regions of homology between heavy γ-chains and light κ- and λ-chains. NH_2 and COOH denote the ends of the chains corresponding to the amino- and carboxyl-terminals, respectively. Data shown in Fig. 10 reveal the homologies between i and i' in the essentially invariant portions of the two chains, whereas the results of affinity labeling experiments strongly suggest the homologies between v and v' in the variant portions. It would appear that the region r on γ-chains is essentially invariant. Those portions of the γ-chain which comprise the Fd and Fc fragments are schematically indicated. The Fc fragment is actually a disulfide-linked dimer of the portion of the heavy chain shown.

that the heavy chains play the primary role and the light chains, a subsidiary role. On the other hand, in many other studies, recovery of substantial binding affinity has occurred only on the recombination of isolated specific heavy and specific light chains of antibodies (*42*). [In one case (*43*), however, nonspecific light chains were reported also to effect the recovery of activity of specific heavy chains.] In a somewhat different type of experiment, specific recombination of antibody heavy and light chains was enhanced by the presence of the hapten specific for that antibody (*44*). Furthermore, some degree of selection of light chains has been demonstrated among antibodies of different specificities (*45*). Such results, therefore, suggest that light chains may be important for antibody specificity. A number of possible structural relationships between the heavy and light chains have been considered (*3, 45*), but no generally accepted view has been derived from the kinds of experiments just mentioned.

On the other hand, the preliminary amino acid sequence data for the Bence Jones proteins indicate that there is potentially great chemical permutability within light chains, and it is difficult to reconcile this information with a subsidiary role for light chains in the determination of antibody specificity.

The results of our affinity-labeling experiments permit us to take a more structural, rather than functional, approach to the problem of heavy and light chains. We have pointed out earlier (*46, 14*) that a chemical similarity exists between the two chains. Our more recent results strongly confirm this suggestion. Consider the following facts or conclusions presented in previous sections. (i) There is a tyrosine residue in a special active-site region on both heavy and light chains of different antibodies. (ii) The labeled tryptic peptides from both chains containing the tyrosine residue from the active sites are of very similar average size, very similar and unusual hydrophobicity, and similar broad chemical heterogeneity. (iii) The labeled chymotryptic peptides from both chains are indistinguishable on paper chromatography and electrophoresis.

These results indicate, first, that heavy chains as well as light chains contain a region which differs in amino acid composition and sequence from one molecule to the next in the population. It is known (*5*) that roughly half of the heavy chain, including the amino (NH_2) terminal end, called the Fd fragment, is involved in the antibody active site, while the other half, the Fc fragment, is not. We conclude, therefore, that there exists a variant region within the Fd fragments of heavy chains as well as within light chains. Frangioni and Franklin have recently reached a similar conclusion from entirely different experiments (*47*).

Beyond this, however, the physicochemical properties of the peptides from these regions of the heavy and light chains are so alike that a strong chemical similarity of these variant regions is indicated. Such a relationship has not been anticipated on chemical grounds in view of well-known differences in the gross properties of heavy and light chains; for example, their molecular weights, overall amino acid composition, NH_2-terminal amino acids, oligosaccharide content, antigenic properties, and total peptide fingerprints differ greatly (*5*). Furthermore, the heavy chain of the IgG molecule is genetically unlinked to the light chain (*48*). However, on the basis of our results, we have further explored the possible relationship between heavy and light chains (*49*).

The nonadecapeptide sequence at the carboxyl (COOH) terminal end of the heavy (γ)-chain of a human IgG myeloma protein has recently been determined by Press *et al.* (*50*). It is shown in Fig. 10*B*, together with the corresponding COOH-terminal sequence of the type-κ Bence Jones light chains (*37, 38*) and the shorter COOH-terminal sequence (*39*) which is at present all that has been determined for type-λ Bence Jones light chains. Although the majority of the amino acid residues are different in corresponding positions along the chains, the number of identities and similarities between the heavy γ- and light κ-chain sequences is clearly significant. These sequences are no less similar than are those of the κ- and λ-chain COOH-terminals, and the κ- and λ-chains, by a number of criteria, are products of related structural genes.

Along similar lines, the amino acid sequences of two related undecapeptides from the heavy γ-chains of two different human IgG myeloma proteins have recently been worked out by Thorpe and Deutsch (*51*). These were derived from the Fc fragments of the heavy chains and are apparently implicated in the different Gm specificities of the two myeloma proteins. These undecapeptides are shown in Fig. 10*A*, together with a decapeptide sequence from the invariant portion of the type-κ Bence Jones light chains (*37, 38*). Here the relationship between the γ- and κ-chains is quite striking. Not only can five of the 11 amino acids be positioned to produce identities between the two peptides, but more particularly each chain contains the tripeptide sequence Pro-Pro-Ser. This is an unusual peptide. In fact, to our knowledge this sequence occurs only in one other case, that of bovine fibrinopeptide *A* (*52*). That it occurs for the two different γ-chains and the three different κ-chains that have so far been examined is therefore highly significant.

The amino acid sequence relationships shown in Fig. 10 are similar to those which exist between proteins which are genetically related, that is,

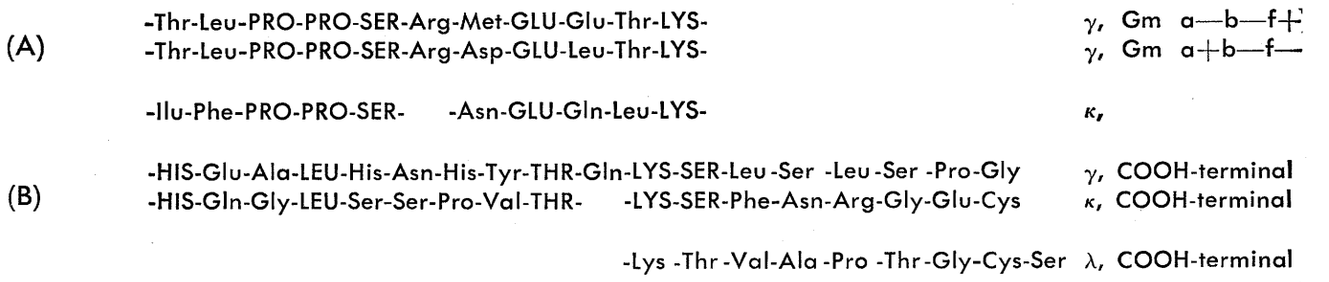


Fig. 10. Partial amino acid sequences of human heavy and light chains. *A*, Two undecapeptides from the heavy γ-chains (*51*), compared with the region of light κ-chains designated 115–124 in *38*; *B*, the carboxyl-terminal regions of the γ-chains (*50*) compared with the carboxyl-terminals of the two light chains (*37–39*).

whose structural genes have arisen from a common ancestral gene but which have undergone independent mutational changes during evolution. Such a genetic relationship between heavy and light chains has not been demonstrated heretofore.

To summarize the discussion of this section up to this point, the results of the affinity labeling studies of rabbit antibodies indicate that a strong chemical similarity exists between a region within the variant half of the light chain and a region within the Fd piece of the γ-chain (regions *v'* and *v*, Fig. 9). Furthermore, the detailed but still fragmentary data just described clearly indicate a chemical relationship between the invariant half of the human κ-chain and the Fc fragment of the human γ-chain (regions *i* and *i'*, Fig. 9). Species differences notwithstanding, the two different halves of the γ-chain therefore both appear to be related to the κ-chain. One possible explanation of these observations is that at some stage in evolution an ancestral gene corresponding to the light chain may have duplicated once and then again, and the two structural genes then fused. This is consistent with the fact that the molecular weight of the γ-chain is approximately twice that of the light chain. On the other hand, there is no evidence that the Fc half of the γ-chain contains a sizable variant region (in position *r*, Fig. 9) of the kind present on the light chains (*v'*) and which we have concluded must also exist on the Fd half of the γ-chain (*v*).

It is evident that the structural considerations presented in this section provide a reasonable answer to the problem of the roles of heavy and light chains in determining the specificity of antibodies. We conclude from our results that heavy and light chains both possess corresponding regions of amino acid sequence which are variable from one molecule to the next in the population; that these regions are involved in the formation of antibody active sites; and that, in fact, the two kinds of chains are related gene products. Since heavy and light chains are so related, both would be expected to participate in the determination of antibody specificity. They need not participate equally, but any inequality would be expected to be more quantitative than qualitative in nature. It may be significant in this connection that the population of labeled tryptic peptides from the γ-chains of antibodies to DNP is not identical with that from the light chains (Fig. 4). The population from the heavy chains is on the average more hydrophobic than that from the light. Such a great similarity but nonidentity of active site regions on the two chains could account for the nonequivalent (2:1, rather than 1:1) labeling of the two chains (Table 1). The absence of specific binding capacity in isolated light chains of antibodies and its retention in the isolated heavy chains may be due to a specific conformational change, or denaturation, that occurs within the active-site regions of isolated light chains but not in isolated heavy chains.

Evolutionary and Biosynthetic Implications

That the γ-chains and κ-chains are genetically related has implications not only for the structure and specificity of the immunoglobulins but also for their evolutionary pathways and the mechanisms of their biosynthesis. Although more detailed chemical studies of the immunoglobulins should shed much light on these relationships, a preliminary discussion of these points may be useful.

Consider, for example, the different heavy chains of the major classes of immunoglobulins (IgG, IgA, and IgM). The different immunoglobulins have common light chains but distinctive heavy chains (γ, α, and μ, respectively) (*53*). There is evidence that, both phylogenetically (*54*) and ontogenetically (*55*), the synthesis of IgM (*19S*) antibody precedes IgG (*7S*). In other words, the heavy μ-chain may be an earlier evolutionary development than the heavy γ-chain. If the COOH-terminal portion of the μ-chain were therefore chemically more closely related to the COOH-terminal region of a light chain than is the COOH-terminal portion of the γ-chain, the μ-chain might retain the cysteine residue at or adjacent to the COOH-terminus which is present on both the light κ- and λ-chains but is missing on the heavy γ-chains (Fig. 10*B*). The presence of such an extra cysteine residue could account for the fact that, in the IgM molecule, five identical subunits (*56*) are linked to one another by disulfide bonds (*57*) to produce a 19*S* molecular species. The possible presence of such a terminal or near-terminal cysteine on the μ-chain is currently being investigated.

The facts that μ- and γ-chains share a common allotypic specificity (*58*) which apparently resides in the Fd fragment of the γ-chain (*59*) and that, on the other hand, μ- and γ-chains differ in the chemistry and antigenicity of their Fc fragments (*5*), have led to the suggestion (*5*) that a heavy chain may actually be made up of two different chains separately synthesized and then covalently bound together. On the hypotheses, however, that the heavy-chain structural gene which first evolved was the result of duplications and fusion of an ancestral light-chain structural gene and that further duplications of the whole heavy-chain gene produced other heavy-chain genes that evolved independently, the properties of μ- and γ-chains may be rationalized. They would then both be single polypeptide

chains, each composed of two similar halves. The NH_2-terminal halves of the two chains may have remained closely similar, while the COOH-terminal halves may have diverged chemically during evolution.

The results presented in this article also bear on the problem of antibody biosynthesis. There have been some rather elaborate speculations recently about mechanisms of antibody biosynthesis, but not enough information is as yet available to provide a sufficiently strong experimental base for an adequate theory. On the other hand, such information is being acquired at a rapid pace from a variety of sources. An adequate theory will have to take into account and be able to explain the structural interrelationship of heavy and light chains which is indicated in this article. A variant region on a light chain and a similar region on a heavy chain must both be generated in the process of formation of an antibody molecule. It appears likely that the variant regions of the heavy γ-chains and the light chains of any particular IgG antibody are not identical but yet are not independent. Their nonidentity is suggested by the nonidentity of the labeled tryptic peptides of the heavy and light chains (Fig. 4), as well as by the different NH_2-terminal residues of the two chains (*5*). Their structural interdependence follows from the conclusion that both chains are involved in the determination of antibody specificity and is supported by a variety of observations, particularly by the finding that homologous pairs of isolated heavy and light chains of myeloma globulin molecules recombine much more efficiently than do heterologous pairs (*60*). In other words, it would appear that, of the potentially very large number of independent permutations of heavy chains and of light chains that might be generated, the heavy and light chains of a particular immunoglobulin molecule may be the result of related permutations. Thus, for example, if it were suggested [as a modification of the clonal-selection theory (*61*)] that multiple somatic mutations in the structural genes which code for the synthesis of the heavy and light chains were responsible for the generation of antibody multiplicity, each antibody-forming cell acquiring one structural gene for a given class of heavy chains and one for light, the possibility would have to be considered that the mutations for the heavy and light chain genes are not independent but are in some manner coordinated, perhaps by a multiple crossing-over between two appropriate zygotic structural genes, one for heavy and one for light chains. A similar mechanism for the coordination of heavy and light chain structure could be postulated for other schemes leading to the generation of antibody multiplicity.

Summary

In order to obtain detailed information about the relationship between structure and function in antibody molecules, a method called affinity labeling has been devised to attach chemical labels specifically to amino acid residues in the active sites of antibody molecules. With antibodies to three different haptens, highly specific labeling of the active sites has been achieved. Tyrosine residues on both heavy and light polypeptide chains have been labeled in a molar ratio close to 2:1, and labels on the two chains are equally specific to the active sites. Peptide fragmentation studies of the labeled chains of one antibody system have shown that: (i) within 25 amino acid residues of the labeled tyrosine on either chain, substantial chemical heterogeneity exists among different antibody molecules of the same specificity; and (ii) the labeled peptide fragments from both chains are very similar in physicochemical characteristics, including average size, heterogeneity, and unusual hydrophobicity.

These experimental results have led us to the view that a particular region of the heavy chain and a particular region of the light chain are utilized to construct the active sites of the three different antibodies, differences in specificity arising from chemical perturbations in these two regions. Correlated structural studies of affinity-labeled antibodies and of the homogeneous light chains (Bence Jones proteins) and heavy chains produced in multiple myeloma may permit the identification of these special active-site regions. The view that active sites of different specificity are chemical perturbations of a particular region of the antibody molecule has a possible close analogue in enzyme systems, particularly among the esterases.

The marked chemical similarities we have observed between the active site regions of heavy and light chains indicate to us that chemical homologies, but not identities, exist between the chains. This is reinforced by recently obtained amino acid sequence data which reveal homologies between the two chains near their carboxyl-terminals. These results indicate that the structural genes which code for the synthesis of heavy and light chains are related, presumably having arisen from some common ancestral gene during evolution. This conclusion strongly suggests that both heavy and light chains determine antibody specificity, and has important implications for the still-unknown mechanisms of antibody biosynthesis.

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64. *Note.* After submission of this manuscript, two items have come to our attention that provide confirmative evidence that a chemical homology exists between the regions v and v' of heavy γ- and light κ-chains (Fig. 9). Hood *et al.* (*62*), by Edman degradations from the NH_2-terminals of seven human Bence Jones κ-chains, have shown that, in confirmation of the work of Hilschmann and Craig (*37*) and Titani *et al.* (*38*), the NH_2-terminal sequence of any one chain is constant but can vary from one chain to another. However, the number of variations at any one position along the chain appears to be limited; shown below are the variations so far found (numerals indicate positions):

1	2	3	4	5	6
Asp,Glu	Ilu	Gln,Val	Val,Met,Leu	Thr	Gln

Porter and Press (*63*) have recently shown that the NH_2-terminal tripeptide of one particular myeloma heavy-γ-chain was PCA-Val-Thr, where PCA is pyrrolidonecarboxylic acid, arising presumably from the cyclization of glutamine. It is of considerable interest that the tripeptide Gln-Val-Thr has its counterpart, therefore, in a possible κ-chain sequence at positions 3, 4, and 5 from the NH_2-terminal.
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Curiosity and Exploration

Animals spend much of their time seeking stimuli whose significance raises problems for psychology.

D. E. Berlyne

Higher animals spend a substantial portion of their time and energy on activities to which terms like *curiosity* and *play* seem applicable (*1*, *2*). An even more conspicuous part of human behavior, especially in highly organized societies, is classifiable as "recreation," "entertainment," "art," or "science." In all of these activities, sense organs are brought into contact with biologically neutral or "indifferent" stimulus patterns—that is, with objects or events that do not seem to be inherently beneficial or noxious. Stimulus patterns encountered in this way are sometimes used to guide subsequent action aimed at achieving some immediate practical advantage. An animal looking and sniffing around may stumble upon a clue to the whereabouts of food. A scientist's discovery may contribute to public amenity and to his own enrichment or fame. Much of the time, however, organisms do nothing in particular about the stimulus patterns that they pursue with such avidity. They appear to seek them "for their own sake."

Until about 15 years ago these forms of behavior were overlooked in the theoretical and experimental literature, except for a few scattered investigations. Recently they have been winning more and more interest among psychologists. They constitute what is generally known in Western countries as "exploratory behavior" and, in Eastern Europe, as "orientational-investigatory activity."

Early demonstrations of the prevalence and strength of these activities in higher animals were rather embarrassing to then current motivation theories. Animals are, of course, most likely to explore and play when they have no emergencies to deal with, but there are times when these behaviors will even override what one would expect to be more urgent considerations. A hungry rat may spend time investi-

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