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A Homologous Series of Affinity Labeling Reagents and Their Use in the Study of Antibody Binding Sites*

P. H. Strausbauch,† Y. Weinstein, M. Wilchek, S. Shaltiel, and D. Givol

ABSTRACT: A series of new radioactive affinity-labeling reagents for anti-DNP antibodies was synthesized. The chemically reactive group in these reagents (bromoacetyl derivatives of DNP haptens) is situated at an increasing distance from the DNP ring. This series thus provides a useful tool for the systematic mapping of antibody binding sites. Each of the reagents reacts specifically with the combining site of anti-DNP antibodies as shown by a variety of chemical and physicochemical criteria, such as stoichiometric bonding to a specific antibody only, and protection of the site by DNP haptens. Using a homologous series of reagents it was possible to show that both tyrosine and lysine become labeled and are apparently present at the combining sites of goat anti-DNP antibodies. The distribution of the label between these two amino acids was different with antibodies from different animals and is probably an individual trait of tne animal.

Affinity labeling of antibodies, initiated by Singer and his colleagues (Wofsy *et al.*, 1962; Metzger *et al.*, 1963), has become an important tool in studying the structural features of the antibody combining site. Studies with diazonium salts of various benzenoid haptens showed that in most cases tyrosines (in both heavy and light chains) are the residues labeled (Singer *et al.*, 1967; Good *et al.*, 1968). An indication that lysine residues might also be present at the combining site of antibodies was obtained by affinity labeling of anti-DNP antibodies with FDNB[1] (Shaltiel and Givol, 1967; Givol *et al.*, 1969). This was strongly supported by the finding that the mouse myeloma protein 315 which has anti-DNP activity could be labeled either at a tyrosine residue in its light chain or at a lysine residue in its heavy chain (Haimovich *et al.*, 1970).

In most of the affinity-labeling reagents, the chemically reactive group can form a covalent bond with only a few of the amino acid side chains that might be present at the binding site. One of the ways to overcome this difficulty was introduced by Fleet *et al.* (1969). These investigators made use of photochemically reactive affinity-labeling reagents which can tag any of the amino acid side chains. In the present study we wish to report another approach, namely the use of an homologous series of reagents in which the chemically reactive group is situated at increasing distances from the haptenic group. Such a series provides a useful tool for locating functional groups at various positions in and around the binding site. This paper describes the synthesis of such an homologous series of reagents, demonstrates the specificity of their reaction, and illustrates the potential uses of such a series for studying the differences in the combining sites of antibodies of the same specificity produced in different individual animals.

Materials and Methods

Synthesis of Reagents. *N*-DNP,*N'*-Z-ETHYLENEDIAMINE. *N*-Z-Ethylenediamine hydrochloride was prepared according to the method of Lawson *et al.* (1968). A solution of 2.3 g (10 mmoles) of *N*-Z-ethylenediamine hydrochloride in 15 ml of water was treated with 2.72 g of FDNB (15 mmoles) in 15 ml of ethanol in the presence of an excess of $NaHCO_3$. The reaction mixture was stirred for 2 hr at room temperature, and the precipitate formed was filtered and washed with water, then with 80% ethanol, and finally with ether. The compound was recrystallized from ethyl acetate: yield, 3 g (83%); mp 133°. *Anal.* Calcd for $C_{16}H_{16}N_4O_6$: C, 53.33; H, 4.44; N, 15.55. Found: C, 53.50; H, 4.53; N, 15.41.

N-DNP-ETHYLENEDIAMINE HYDROBROMIDE. A solution of 1.8 g (5 mmoles) of *N*-DNP-*N'*-Z-ethylenediamine in 10 ml of glacial acetic acid was mixed with 15 ml of HBr in CH_3COOH (45%). The reaction was allowed to proceed for 15 min, then stopped by addition of dry ether. The precipitate was washed with dry ether: yield, 1.4 g (90%); mp over 250°. *Anal.* Calcd for $C_8H_{11}BrN_4O_4$: C, 31.28; H, 3.61; N, 18.24. Found: C, 31.50; H, 3.40; N, 17.97.

N-BROMOACETYL-*N'*-DNP-ETHYLENEDIAMINE (BADE) (See Chart I). *N*-DNP-ethylenediamine hydrobromide (0.77 g, 2.5 mmoles) was suspended in 10 ml of dioxane containing 2.5 ml of 1 N NaOH and 5 ml of 1 N $NaHCO_3$ was added. This mixture was reacted with 0.6 g (2.5 mmoles) of *N*-hydroxysuccinimide ester of bromoacetic acid (Cuatrecasas *et al.*, 1969) which was dissolved in dioxane. The reaction was

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[1] Abbreviations used are: FDNB, fluorodinitrobenzene; BADE, *N*-bromoacetyl-*N'*-DNP-ethylenediamine; BADL, N^{α}-bromoacetyl-N^{ϵ}-DNP-lysine; BADB, N^{α}-bromoacetyl-N^{γ}-DNP-diamino-L-butyric acid; BADO, N^{α}-bromoacetyl-N^{δ}-DNP-L-ornithine; IADL, N^{α}-iodoacetyl-N^{ϵ}-DNP-L-lysine; Z, benzyloxycarbonyl.

allowed to proceed for 20 min; then the reaction mixture was acidified and the product extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4, and concentrated to dryness. Upon trituration with ether the compound crystallized and it was then recrystallized from ethyl acetate. The yield was 0.65 g (75%), mp 163°. *Anal.* Calcd for $C_{10}H_{11}BrN_4O_5$: C, 34.60; H, 3.19; N, 16.14. Found: C, 34.35; H, 3.32, N, 16.30.

N^{δ}-DNP-L-ORNITHINE HYDROCHLORIDE. A sample of L-ornithine hydrochloride (1.68 g, 10 mmoles) was dissolved in 15 ml of boiling water and 2 g of $CuCO_3$ was added with stirring over a period of 10 min. The excess of $CuCO_3$ was removed by filtration, and after washing with 5 ml of hot water the filtrate was collected. To the blue filtrate we added an excess of $NaHCO_3$ and a solution of 3.6 g of FDNB in 20 ml of ethanol. The reaction mixture was stirred for 2 hr at room temperature. The green precipitate formed was filtered, washed successively with water, ethanol, and ether, and then dissolved in 10 ml of 3 N HCl. After about 10 min a yellow precipitate was formed which was collected and washed with 5 ml of cold 3 N HCl, 5 ml of cold water, and then ethanol and ether: yield, 2.35 g (70%); mp 258°. *Anal.* Calcd for $C_{11}H_{15}ClN_4O_6$: C, 39.40; H, 4.47; N, 16.71. Found: C, 39.55; H, 4.53; N, 16.54.

N^{δ}-DNP-N^{α}-BROMOACETYL-L-ORNITHINE (BADO). N^{δ}-DNP-L-Ornithine hydrochloride (1.68 g, 5 mmoles) was dissolved in cold water containing 2 equiv of NaOH. To this solution, 3 equiv of $BrCH_2COBr$ were added and the reaction was allowed to proceed at 0° for 20 min, keeping the pH > 8 with $NaHCO_3$. The reaction mixture was left for another 10 min at room temperature, then acidified to pH 2 with HCl, and the product was extracted into ethyl acetate. The extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated to dryness. After recrystallization from ethyl acetate–petroleum ether (bp 30–60°) the yield was 1.7 g (80%); mp 157–159°. *Anal.* Calcd for $C_{13}H_{15}BrN_4O_7$: C, 37.24; H, 3.61; N, 13.37. Found: C, 37.51; H, 3.82; N, 13.13.

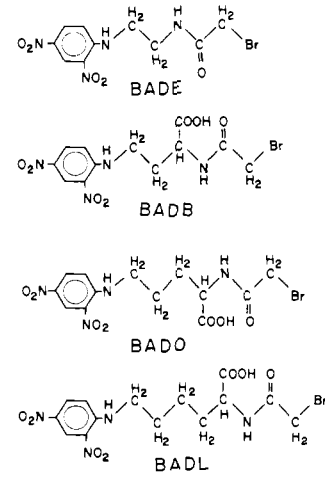
N^{γ}-DNP-N^{α}-BROMOACETYL-L-DIAMINOBUTYRIC ACID (BADB). This compound was prepared from N^{γ}-DNP-L-diaminobutyric acid hydrochloride (Givol *et al.*, 1971)[2] and $BrCH_2COBr$ as described for BADO: yield, 75%; mp 81°. *Anal.* Calcd for $C_{12}H_{13}BrN_4O_7$: C, 35.57; H, 3.23; N, 13.83. Found: C, 35.37; H, 3.73; N, 14.09.

N^{ϵ}-DNP-N^{α}-BROMOACETYL-L-LYSINE (L-BADL) and N^{ϵ}-DNP-N^{α}-BROMOACETYL-D-LYSINE (D-BADL) were prepared as described by Weinstein *et al.* (1969).

N^{ϵ}-DNP-N^{α}-IODOACETYL-L-LYSINE (IADL) was prepared as described by Brenneman and Singer (1970).

Radioactivity labeled reagents were prepared by a scaled down modification of the procedure for the preparation of the corresponding nonradioactive reagent. Solutions of 1.2 mg of *N*-hydroxysuccinimide (13 μmoles) in 0.1 ml of ethyl acetate and 2.8 mg of dicyclohexylcarbodiimide (14 μmoles) in 0.1 ml of dioxane were mixed with 0.1 ml of a stock solution of [^{14}C]$BrCH_2COOH$ (10 μmoles, 4.1 μCi/μmole (in dioxane), Amersham) and allowed to react for 3 hr at 25°. The precipitate of dicyclohexylurea was removed by filtration through cotton wool in a Pasteur pipet, and the filtrate was added immediately to a solution of the appropriate DNP compound (10 μmoles) in 1 ml of 50% aqueous dioxane containing 10 mg of $NaHCO_3$. After 1 hr at 25° the solution was acidified, the [^{14}C]bromoacetyl-DNP reagent was extracted into ethyl acetate, and the extract was evaporated to dryness. The reagent was further purified by thin-layer chromatography on silica gel (Riedel-DE Haen AG) developed with chloroform–*tert*-amyl alcohol–acetic acid (70:30:3 v/v). The radioactive reagents were located by markers of the purified nonradioactive reagents and then eluted with dioxane. The R_F values of the various reagents were found to be: BADE, 0.72; BADB, 0.58; BADO, 0.72; D- and L-BADL, 0.80; and IADL, 0.78.

CHART I



Antigens. The protein antigens used for immunization were prepared by dinitrophenylation with an equal weight of DNP-sulfonate under the conditions described by Eisen *et al.* (1953).

Antibodies. Rabbits were immunized with DNP-bovine serum albumin and goats with DNP-keyhole limpet hemocyanine. All animals were immunized by two injections, 3 weeks apart. Each injection consisted of 1 mg of the antigen in 1 ml of a buffer composed of 0.15 M NaCl and 0.01 M sodium phosphate (pH 7.4) emulsified with 1 ml of complete Freund's adjuvant (Difco), and injections were given at multiple intradermal sites. Animals were bled weekly after the second injection and when antibody titers decreased to below 0.5 mg/ml they were boosted by reinjection in the same manner. Rabbit antisera were pooled whereas the antiserum of each bleeding from each goat was kept separate. Anti-DNP antibodies were isolated on a DNP rabbit serum albumin–Sepharose immunoadsorbent prepared according to Porath *et al.* (1967) as described by Givol *et al.* (1970). The adsorbed antibodies were eluted by incubation (1 hr) with 0.1 M acetic acid at 37°, then dialyzed against a buffer composed of 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4). The myeloma protein 315 (a mouse IgA possessing anti-DNP activity, Eisen *et al.*, 1968) was isolated from the serum of tumor bearing mice by the procedure described by Haimovich *et al.* (1970). The number of binding sites of all the above preparations was determined by fluorescence quenching with N^{ϵ}-DNP-L-lysine (Eisen and Siskind, 1964). The average number of sites per antibody molecule were: 1.60 for the rabbit antibodies, 1.30 for goat 8, 0.95 for goat 36, 1.52 for goat 44, and 1.60 for the myeloma protein 315. These values were taken as 100% of the available sites in affinity-labeling experiments. Antibody concentrations were determined spectrophotometrically using absorbancy indexes ($A^{1\%}_{280\ nm}$) of 14.0 and 13.0 for rabbit and goat antibodies, respectively (Crumpton and Wilkinson, 1963; Givol and Hurwitz, 1969), and 14.0 for the myeloma protein 315 (Eisen *et al.*,

[2] Submitted for publication.

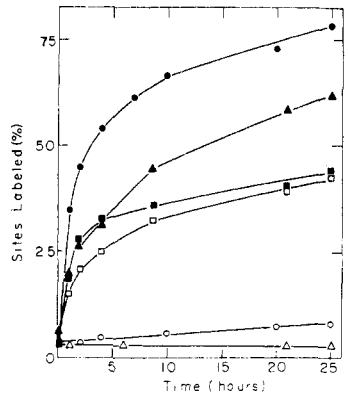


FIGURE 1: Rate of reaction between pooled rabbit anti-DNP antibodies and various homologous DNP reagents: BADE (●), BADO (□), and BADL (▲). Controls with normal rabbit immunoglobulin G: BADE (○) and BADL (△).

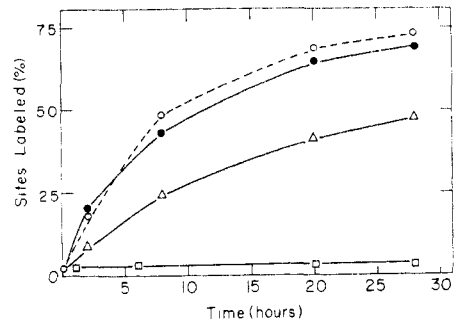


FIGURE 2: Reaction of pooled rabbit anti-DNP antibodies with BADL under different conditions: molar ratio of BADL to protein 20:1 (○); molar ratio of BADL to protein 2:1 (●); molar ratio of BADL to protein 2:1 in the presence of equimolar amount of N^{ϵ}-DNP-aminocaproate (△); control with normal rabbit immunoglobulin G using molar ratio of BADL to protein of 2:1 (□). Protein concentration was 0.15 mg/ml and the reaction was conducted in 0.1 M $NaHCO_3$, pH 9.0, 37°.

1968). The molecular weights of all immunoglobulins were taken as 150,000.

Affinity Labeling of Antibodies. Unless otherwise mentioned, affinity labeling of immunoglobulins was carried out in a reaction mixture composed of antibody (1×10^{-6} M), the ^{14}C affinity-labeling reagent (4×10^{-6} M), and 0.1 M $NaHCO_3$ (pH 9.0) (a ratio of labeling reagent to binding sites of two to one since each mole of antibody contains 2 moles of hapten binding sites). The reaction was allowed to proceed at 37° and the extent of covalent bonding at various times was determined by precipitation of the protein with 25% CCl_3COOH, filtration on selectron filters (Weinstein *et al.*, 1969), and counting the radioactivity. Upon acid hydrolysis (6 N HCl, 108°, 24 hr) the labeled amino acids yield the corresponding CM derivatives. The [^{14}C]CM-amino acids were identified by paper electrophoresis (4000 V, pH 3.5). Nonradioactive *O*-CM-tyrosine and N^{ϵ}-CM-lysine were added to the acid hydrolysates as internal markers. The electrophorogram was stained with ninhydrin and the spots containing the above markers were traced. After bleaching the paper with acidic acetone (1 ml of 1 N HCl in 40 ml of acetone) the traced spots were cut and counted. The accuracy of these determinations was confirmed by analyses on an amino acid analyzer to which a scintillation flow cell was attached (Weinstein *et al.*, 1969). In all the experiments reported here only tyrosine and lysine were found to be labeled.

Analytical separation of heavy and light chains of immunoglobulins was performed by polyacrylamide gel electrophoresis using 6% gels in 0.1% sodium dodecyl sulfate and 0.14 M 2-mercaptoethanol (Shapiro *et al.*, 1967). The staining of the gels was performed with coomassie brilliant blue (Chrambach *et al.*, 1967). Radioactive bands in the gels were located by cutting the gel into thin slices which were then solubilized by addition of 1 ml of Soluene (Packard Instrument Co.) and incubation for 12 hr at 37°. Protein bands were analyzed for [^{14}C]-CM-amino acids by acid hydrolysis (6 N HCl, 108°, 24 hr) performed directly on the thin slices of gel. The hydrolysates were cooled to 0°, centrifuged to remove the residual polyacrylic acid, dried, and analyzed by high-voltage paper electrophoresis as described above. Standard samples of radioactively labeled protein which were run under the above conditions showed that the recovery of labeled amino acids is quantitative.

Markers of N^{ϵ}-CM-lysine and O-CM-tyrosine. N^{ϵ}-CM-lysine was prepared by reacting polylysine (n = 180, Yeda) with iodoacetate and subsequent hydrolysis (Gundlach *et al.*, 1959). The marker was purified by high-voltage electrophoresis at pH 3.5. N^{ϵ}-CM-lysine moved as a neutral amino acid (mobility relative to Asp = −0.02). *O*-CM-tyrosine was prepared as follows: a solution of *N-tert*-butyloxycarbonyltyrosine (1.6 g in 10 ml) was reacted with 2 g of bromoacetic acid and maintained at pH 10.6 by addition of 5 M NaOH. The reaction was allowed to proceed for 12 hr at 24°; then the mixture was acidified to pH 1 with HCl. The product, *N-tert*-butyloxycarbonyl-*O*-CM-tyrosine, was extracted into ethyl acetate and the extract was dried with anhydrous Na_2SO_4, then evaporated to dryness. The oily residue was dissolved in 5 ml of anhydrous CF_3COOH and allowed to stand for 30 min at 25°. Upon addition of 250 ml of ether *O*-CM-tyrosine and tyrosine precipitated. These two products were separated by preparative paper electrophoresis at pH 3.5. The mobility of *O*-CM-tyrosine relative to Asp was −1.31. Both markers were checked for purity on the amino acid analyzer. *O*-CM-Tyrosine emerged from the column between Val and Met and N^{ϵ}-CM-lysine immediately after Met.

Instruments. Absorption measurements were taken with a Cary spectrophotometer Model 15 and fluorescence studies were carried out with a Turner 210 "Spectro." Counting of radioactive samples was performed with a Packard Model 3003 Tri-Carb liquid scintillation spectrometer.

Results

The homologous series of affinity-labeling reagents used in this study comprises four reagents.

Restricted Bonding of the Reagents to the Combining Site of Antibodies. Each of the affinity-labeling reagents reacts specifically with the binding sites of rabbit anti-DNP antibodies. As seen in Figure 1, the rate of covalent bonding of the various reagents to the antibody is rather slow, reaching completion (45–80% of the sites) in several hours. Nevertheless, the specificity of the reaction is preserved, as indicated by the fact that with normal immunoglobulin essentially no covalent bonding takes place (Figure 1). The rate and extent of covalent labeling of the antibodies do not increase even when a tenfold excess of the labeling reagent is used (Figure 2). On the other hand, DNP haptens such as N^{ϵ}-DNP-lysine or N^{ϵ}-DNP-aminocaproic acid inhibit the covalent attachment of BADL to the antibodies and the extent of protection afforded by the hapten is a function of its concentration (Figures 2 and 3).

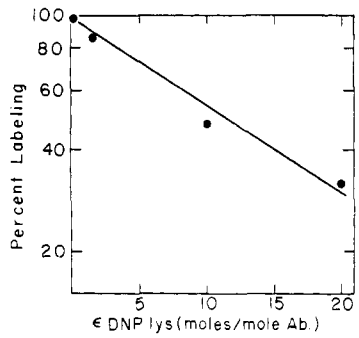


FIGURE 3: Protection by hapten against affinity labeling of antibodies. Rabbit anti-DNP antibodies were reacted with BADL (pH 9.0, 37°) in the presence of increasing amount of N^{ϵ}-DNP-L-lysine. The amount of covalently attached reagent was determined after 15-hr reaction.

Spectral studies confirm that the affinity-labeling reagent BADE, after reacting covalently, still occupies the anti-DNP binding site. This is indicated by the characteristic red shift in the spectrum of DNP groups that occurs upon their binding to anti-DNP antibodies (Little and Eisen, 1967). Complex formation between BADE and the combining site of the antibodies brings about this red shift even before covalent bonding (Figure 4, curve 1). This shift is preserved after covalent bonding (Figure 4, curve 3), indicating that there is no dislocation of the DNP group as a result of covalent bond formation. Moreover, if BADE is added to normal rabbit IgG (2 moles/mole) and the reaction is carried out at high protein concentration (10 mg/ml) some covalent bonding of BADE to normal rabbit IgG does occur (0.17 mole/mole IgG) but there is no red shift of the DNP spectrum (Figure 4, curve 2).

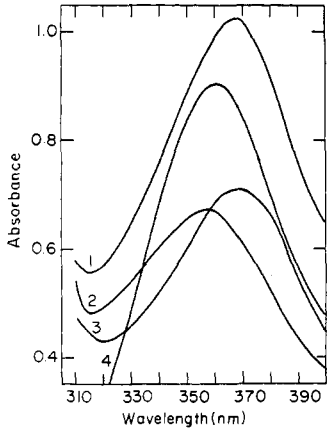


FIGURE 4: Adsorption spectra of free BADE and BADE bound to immunoglobulins. Curve 1, BADE (6.65×10^{-5} M) noncovalently bound to rabbit anti-DNP antibodies (3.70×10^{-5} M). Spectrum was taken 10 min after mixing of reagents. Curve 2, BADE nonspecifically linked to normal rabbit immunoglobulin G by reaction with concentrated reagents (BADE 1.2×10^{-4} M; immunoglobulin, 6.6×10^{-5} M) for 48 hr, followed by concentration and dialysis against 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4). Concentration of species in spectrum (covalently bound DNP, 3.75×10^{-5} M; immunoglobulin, 2.25×10^{-4} M). Curve 3, purified rabbit anti-DNP antibodies affinity labeled with BADE as described in Materials and Methods. Concentration of species in spectrum (covalently bound DNP, 4.55×10^{-5} M; immunoglobulin, 3.18×10^{-5} M). Curve 4, spectrum of BADE alone (5.35×10^{-5} M). All spectra were taken in 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4).

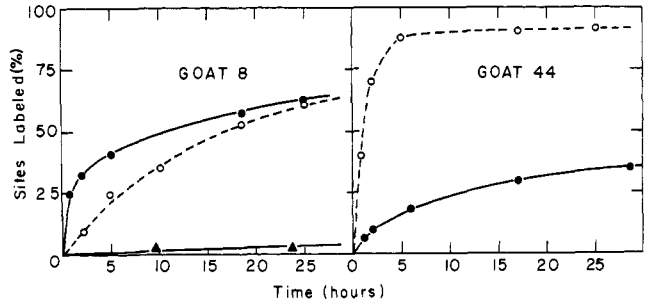


FIGURE 5: Rate of reaction between various labeling reagents and anti-DNP antibodies or normal immunoglobulin. Reaction of BADE (●) and BADO (○) with purified anti-DNP antibodies from individual goats; reaction of BADE (▲) with normal goat immunoglobulin. For reaction conditions, see Materials and Methods.

Reaction of Goat Antibodies with the Labeling Reagents. Reaction of antibodies from different individual goats with the various labeling reagents revealed remarkable differences both in the rate and in the extent of covalent labeling. For example, antibodies from goat 8 reacted more rapidly and to a larger extent with BADE than with BADO while with goat 44 the picture was reversed (Figure 5). Further details about the behavior of antibodies with the various reagents are given in Table I. With goat 8 the rate of reaction is maximal with BADE and decreases with increasing the size of the reagent. On the other hand, with antibodies from goat 44 the rate increases with increasing size until the size of BADO is reached. Further increase in the size of the reagent results in a

TABLE I: Labeling of Goat Anti-DNP Antibodies with Affinity-Labeling Reagents.[a]

Antibody Prepn	Reagent	Extent of Reaction[b]	Half-Life Time of Reaction (hr)[c]
Goat 8	BADE	1.20	6.5
	BADB	1.60	7.0
	BADO	1.17	15.5
	L-BADL	0.96	26.0
	D-BADL	1.30	15.0
Goat 36	BADE	0.61	45.0
	L-BADL	0.62	30.0
Goat 44	BADE	0.64	58.0
	BADB	0.84	41.0
	BADO	1.81	1.5
	L-BADL	0.96	25.5
	D-BADL	1.30	9.0
	IADL	0.84	30.0

[a] Reaction conditions: 1 mμmole/ml of antibody in 0.1 M $NaHCO_3$, pH 9.5, 37° was reacted with 4 mμmoles/ml of affinity-labeling reagent. [b] Extent of reaction is given as moles of radioactive reagent covalently bound per 2 moles of hapten combining sites after 24-hr reaction. Actual number of combining sites was determined by fluorescence quenching (see Materials and Methods). [c] Time required for covalent labeling of 50% of the available sites.

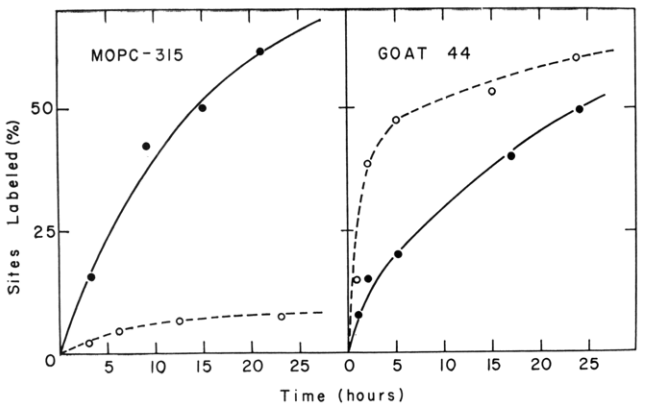


FIGURE 6: Stereospecificity in the labeling of myeloma protein 315 and anti-DNP antibodies from goat 44. Both proteins were reacted with either the D (○) or L (●) isomers of BADL under the conditions described in Materials and Methods.

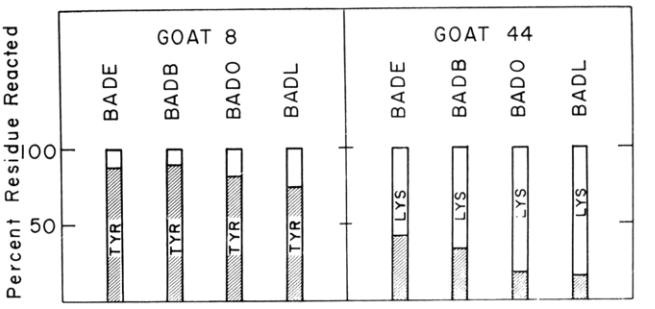


FIGURE 8: Covalent labeling of anti-DNP antibodies with BADE, BADB, BADO, and BADL. Each of the labeled antibody was hydrolyzed and analyzed for CM-amino acids. The upper part of each bar (unshaded) gives the percentage of labeled CM-lysine, and the lower part (shaded) of each bar gives the percentage of CM-tyrosine. The extent of labeling of the antibodies by each reagent is given in Table II.

decrease in the rate of reaction. Another manifestation of the variability in behavior of various antibodies is reflected in the differences observed with two disastereoisomers of the same reagent (L- and D-BADL). For example, the antibodies from goat 44 react with the D isomer faster than with the L isomer (Figure 6). An even more accentuated stereospecificity was found in the case of the myeloma protein 315 (Figure 6) which dramatically differentiates between the two diastereoisomeric reagents and essentially reacts only with the L isomer (Givol *et al.*, 1971).[2]

Amino Acids and Peptide Chains Modified by Labeling Reagents. In contrast to the pooled rabbit antibodies, in which 95% of the label was on tyrosyl residues (Weinstein *et al.*, 1969), goat antibodies are labeled both on tyrosines and lysines. However, the distribution of the label between these two amino acids varied markedly in antibodies from different animals (Figure 7). As seen in Figure 8, the antibodies obtained from goat 8 were labeled mostly on tyrosine while in goat 44 most of the label was found on lysine. It should be noted that in the antibodies of both goats there appears to be a tendency for an increased labeling of lysine with increasing length of the reagent (Figure 8), yet this tendency is minor relative to the marked individual difference between the two goats.

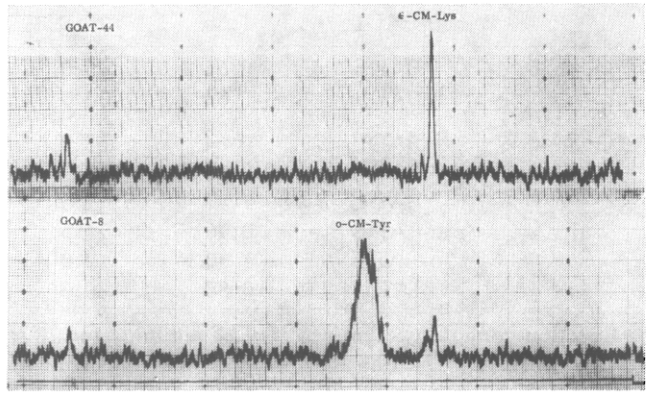


FIGURE 7: Analysis of the radioactively labeled CM-amino acid residue obtained after acid hydrolysis of anti-DNP antibodies. Top figure, antibodies from goat 44 labeled with BADL. Bottom figure, antibodies from goat 8 labeled with BADE. An amount of the hydrolysate which contained 10,000–20,000 cpm was applied to the long column of the amino acid analyzer. Radioactivity was monitored using a Packard scintillation flow cell attached to the analyzer.

Since the above studies were conducted on purified antibody preparations isolated from single bleedings, it was of interest to determine whether the different labeling patterns observed with individual animals were due to fortuitous selection of samples or to a real individual trait, preserved throughout the animal's life. Antibodies from bleedings made at various times over a period of 18 months showed a fairly consistent distribution of the label among tyrosine and lysine, characteristic to each goat (Figure 9). This trait was preserved even after additional immunization.

These results could be explained by assuming that, with respect to these reagents, there are at least two populations of antibodies within each goat: one which reacts preferentially through its tyrosines and the other through its lysines. As seen in Figure 10, the ratio of labeled tyrosine to labeled lysine changes in the course of labeling. If the distribution of the label between these two amino acids was simply a reflection of their relative reactivities within one molecule, a constant ratio of labeled tyrosine and lysine would be expected. The results depicted in Figure 10 clearly exclude this possibility, favoring the existence of two antibody populations.

Upon tagging of the goat antibodies with various reagents, the label was found to be distributed in all cases between the heavy and the light chains. However, this distribution was different with different reagents and also with different animals. As seen in Table II, when goat 8 was treated with BADE

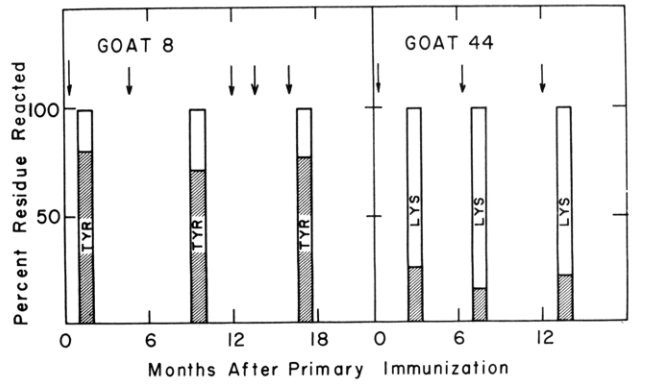


FIGURE 9: Affinity labeling of goat anti-DNP antibodies isolated at different times following immunization. Bar graphs indicate the percentage of lysyl (unshaded) and tyrosyl (shaded) residues labeled by BADE. Initial immunization was made at time zero. Arrows mark booster injections.

TABLE II: Distribution of Label on Affinity Labeled Anti-DNP Antibodies.[a]

Antibody Prepn	Reagent	Ratio of Label in Heavy Chain to That in Light Chain	Labeled Residue (%)					
			Whole IgG		Heavy chain		Light chain	
			CM-Lys	CM-Tyr	CM-Lys	CM-Tyr	CM-Lys	CM-Tyr
Goat 8	BADE	1.1:1	12	88	20	80	11	89
Goat 8	L-BADL	1.7:1	28	72	34	66	43	57
Goat 44	BADE	1.7:1	59	41	70	30	52	48
Goat 44	L-BADL	1.9:1	76	24	85	15	72	28

[a] Immunoglobulin (1 mg/ml in 0.1 M $NaHCO_3$, pH 9.0) was incubated with a twofold excess of labeling reagent for 24 hr, 37°. The distribution of label between heavy and light chains as well as between CM-tyrosine and CM-lysine was determined as described in Materials and Methods.

the tag was found in the heavy and light chains with a ratio of 1.1:1. On the other hand, when the same antibodies were treated with BADL, a higher proportion of the label was directed to the heavy chain (ratio 1.7:1). Also when BADE is used for tagging antibodies from goat 44, a ratio of 1.7:1 is found, compared with 1.1:1 for goat 8. These results were confirmed by preparative separation of heavy and light chains (from reduced and alkylated antibodies) on a Sephadex G-100 column, equilibrated with 8 M urea containing 1 M propionic acid. The distribution of the tag between lysine and tyrosine in the heavy chain and in the light chain is similar (though not identical) with the average distribution found in the intact antibody (Table II).

Discussion

A homologous series of affinity-labeling reagents, in which the chemically reactive group is situated at increasing distances from the antigenic determinant, provides a means for systematic mapping of the antibody binding site and its vicinity. As in other studies on affinity labeling of antibodies (Wofsy *et al.*, 1962; Singer, 1967) special care was taken to ensure that each reagent in the series is indeed site specific. This was demonstrated by the following findings: (a) the reaction was stoichiometric, restricted to specific antibodies, and negligible with normal immunoglobulins. (b) The rate and extent of the reaction were not enhanced by increasing the excess of labeling reagent, indicating that covalent bonding takes place within the specific complex that is formed between the antibody and the reagent. (c) Specific haptens protect the antibodies from labeling by competing with the labeling reagents for the vacant sites of the antibody. Moreover, upon increasing the hapten concentration, the rate of labeling decreases. (d) After covalent attachment of the labeling reagent to the antibody there is no dislocation of the haptenic group and the red shift in the absorption spectrum (which is characteristic of DNP haptens bound to anti-DNP antibodies) is preserved. All these data, together with the fact that upon covalent bonding there is a loss of antibody sites proportional to the extent of labeling (Weinstein *et al.*, 1969), strongly indicate that the labeling occurs either at the binding site itself or in its immediate vicinity.

The largest reagent used (BADL) is about 17 Å long in its fully stretched conformation. This is within the size range that has been suggested for the antibody combining site (Kabat, 1968). Therefore, it is very probable that tagging occurs inside the binding site. Furthermore, reagents of the type used here may assume a variety of conformations, and the particular conformation while in the binding site may be dictated by the structure of the site. This conformation need not necessarily be the fully stretched one. The recent demonstration (S. Haimovich, personal communication) that BADE tags the myeloma protein 315 at the same residue that is labeled with the diazonium salt of *m*-nitrobenzene (Goetzl and Metzger, 1970) indicates that the conformation of this reagent (BADE) within the site may indeed not be the fully stretched one, and that such reagents may label "contact" amino acid residues. In any case it is conceivable that as the functional group in the labeling reagent becomes more and more removed from the DNP ring, one is bound to label other residues in the vicinity of the site. Nevertheless, as long as the reaction is specifically directed by the antibody site, any labeled residue may provide important information.

Previous affinity-labeling studies have indicated that tyrosine and in some cases histidine are present in the combining site of antibodies (Singer *et al.*, 1967; Wofsy and Parker, 1967). We have recently shown that FDNB labels both tyrosine and lysine residues in rabbit anti-DNP antibodies (Shaltiel and

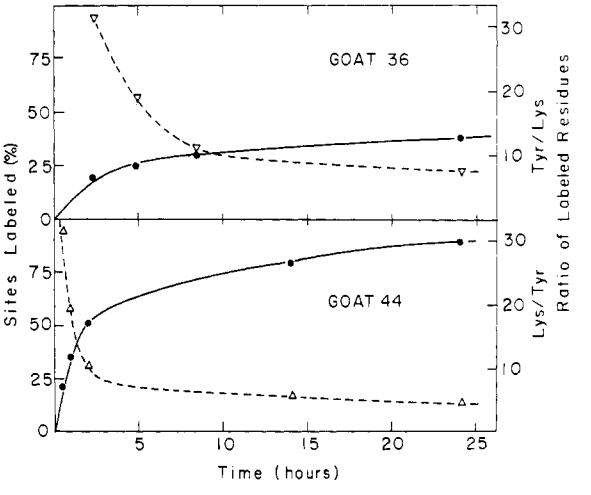


FIGURE 10: Variation in the ratio of labeled amino acid residues as a function of reaction time. Upper graph: per cent of sites labeled (●) and ratio of labeled tyrosyl:lysyl residues (▽) in the reaction of goat 36 anti-DNP antibodies with BADE. Lower graph: per cent of sites labeled (●) and ratio of lysyl:tyrosyl residues (Δ) in the reaction of goat 44 anti-DNP antibodies with BADO.

Givol, 1967). From the heavy chain of these antibodies a labeled peptide with the sequence Ala-(ϵ-DNP)Lys was isolated (Givol *et al.*, 1969). The possible involvement of lysine residues was also supported by the finding that BADL tagged a specific lysyl residue in the heavy chain of the myeloma protein 315 (Haimovich *et al.*, 1970). In the present study with goat antibodies, lysine was labeled in all cases and with all the reagents used. In one antibody preparation (from goat 44) lysine was the predominant amino acid residue to be tagged. Of course, the possibility exists that this lysine residue might not be at a "contact distance" from the DNP group. In this connection it should be mentioned that the extent of lysine labeling increased slightly with increasing size of the reagent. However, in goat 44, lysine was the predominant residue to be labeled even with BADE, the smallest reagent.

Since the chemical reactivity of the bromoacetyl group in the various reagents is probably very similar, the differences observed in the rate and extent of labeling with various antibodies must be attributed to the geometric positioning of the reagent within the site. The preferential labeling of antibodies and of the myeloma protein 315 by one diastereoisomer rather than the other strongly supports this hypothesis.

One of the interesting observations made in the course of this study was the occurrence of differences in the distribution of the label in antibodies from different individual animals of the same species (see also Koyama *et al.*, 1967). The characteristic distribution of the label between tyrosine and lysine appears to be preserved throughout the life of the animal as an individual trait. Since this difference in distribution is most probably a reflection of the amino acid sequence in the variable regions of the heavy and light chains, it may provide genetic markers for following differences in the inheritance of different sequences in the variable regions of immunoglobulins.

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