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Affinity Labeling of a Mouse IgG2a Myeloma Protein with Binding Affinity for Nitrophenyl Ligands†

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ABSTRACT: A nitrophenyl-binding IgG2a myeloma protein, HPC-3, has been affinity labeled with the reagent [^{3}H]*m*-nitrobenzenediazonium fluoborate under conditions previously used with anti-2,4-dinitrophenyl (Dnp) antibodies. Specific affinity labeling of the active sites of HPC-3 protein was produced, by the criterion that two to three times as much radioactivity was covalently bound to the protein in the absence as in the presence of an excess of the reversibly bound protector, Dnp-aminocaproate. The specific label was predominantly in the form of *m*-nitrobenzeneazotyrosine. About 90% of the affinity label was bound to the H chains of HPC-3 protein, but the 10% of label attached to L chains was also at least partly specific for the active sites. These affinity-labeling results are similar to those obtained with mouse anti-Dnp antibodies, but by several criteria, the active sites of HPC-3 protein and of elicited anti-Dnp antibodies can be distinguished.

Affinity labeling has been applied to the study of the active sites of a variety of anti-hapten antibodies (*cf.* Singer *et al.*, 1967; Wofsy *et al.*, 1967). Detailed structural investigations of the labeled antibodies have been successful to some extent (Thorpe and Singer, 1969; Cebra *et al.*, 1971), although they are made difficult by the gross molecular heterogeneity of most antihapten antibody preparations, even from an individial animal. Myeloma proteins are usually homogeneous immunoglobulins, some of which have been shown to bind certain small molecules with considerable specificity (Eisen *et al.*, 1967, 1970; Potter and Leon, 1968; Cohn *et al.*, 1969). It is clearly of interest to attempt to affinity label these ligand-binding myeloma proteins, and to assess the relevance of the results obtained to those for affinity-labeled elicited anti-hapten antibodies. Metzger and Potter (1968) showed that the mouse IgA[1] myeloma protein MOPC-315, with binding affinity for Dnp[2] ligands, could be affinity labeled with the same specific diazonium reagents used to label elicited anti-Dnp antibodies (Metzger *et al.*, 1963; Good *et al.*, 1967). Further affinity labeling studies with MOPC-315, using other types of reagents, have been reported (Haimovitch *et al.*, 1970). In this paper, we report the results of affinity labeling of a mouse IgG2a myeloma protein, HPC-3 (Warner and Ovary, 1970) with the diazonium reagent [^{3}H]MNBDF. There is specific affinity labeling of the active site of the protein at tyrosine residue(s) only. The specific label is predominantly on the H chain of the protein, but the small amount of label on the L chain appears also to be at least partly specific. The significance of these and other results in assessing the relationship between hapten-binding myeloma proteins and elicited anti-hapten antibodies is discussed.

Materials and Methods

The induction of the plasma cell tumor HPC-3 in inbred NZB mice, and isolation and partial characterization of the pure HPC-3 myeloma protein has been described (Warner and Ovary, 1970). The mouse anti-Dnp antibodies were raised in Swiss-Webster mice by immunization with Dnp-hemocyanin, and the pooled antibodies were isolated, according to published procedures (Thorpe and Singer, 1969).

For most of the experiments reported in this paper, the HPC-3 protein was reacted with the affinity-labeling reagent [^{3}H]MNBDF under the same conditions used in earlier experiments with rabbit and mouse anti-DNP antibodies (Good *et al.*, 1967; Thorpe and Singer, 1969), with or without a 13-fold or 100-fold excess of the specific protector, *N*-Dnp-ε-aminocaproate. For spectral analyses of the products of the affinity-labeling reaction, nonradioactive MNBDF was used under the same conditions. For all the labeling experiments with HPC-3 protein, parallel experiments were carried out with a pooled preparation of pure mouse anti-Dnp antibodies.

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[1] Nomenclature for immunoglobnlins corresponds to that recommended by the World Health Organization (*Bull. W.H.O. 30*, 447 (1964)).

[2] Abbreviations used are: Dnp, 2,4-dinitrophenyl; MNBDF, *m*-nitrobenzenediazonium fluoroborate; DnpNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonate.

TABLE I: Affinity Labeling with [^{3}H]MNBDF.[a]

Protein	Reaction Condition	Whole Protein	H Chains	L Chains	2 H + 2 L
Mouse anti-Dnp-antibodies	Unprotected	0.360	0.108	0.123	0.462
	Protected[b]	0.064	0.020	0.015	0.069
HPC-3	Unprotected	0.730			
	Protected[b]	0.253			
	Unprotected	0.615	0.250	0.027	0.554
	Protected[b]	0.177	0.065	0.015	0.159
	Unprotected	0.770			
	Protected[c]	0.406			

[a] Data expressed as mol of label bound per mol of protein, taking the molecular weights of antibodies and HPC-3 protein as 160,000, H chains 55,000, and L chains 25,000. [b] Reaction in presence of a molar concentration of Dnp-aminocaproate 13 times that of protein. [c] Reaction in presence of a molar concentration of Dnp-aminocaproate 100 times that of protein.

After the 6-hr reaction time at pH 5.0, the radioactively labeled samples of HPC-3 and mouse anti-Dnp antibodies were brought to pH 4.0 with glacial acetic acid to stop the reaction and were then dialyzed against 0.2 M sodium acetate–acetic acid buffer (pH 4.0) to remove most of the noncovalently bound radioactivity. The samples were then dialyzed against 0.01 M Tris buffer (pH 8.2). In this medium, the proteins were then succinylated in order to facilitate the subsequent separation of their H and L chains (Lenard and Singer, 1966). Succinylation was carried out at 0° for 1.5 hr employing a 5-fold molar excess of solid succinic anhydride over the estimated free amino groups of the proteins, at protein concentrations of 2–2.5 mg/ml. The pH of the reaction was maintained at ~8.2 by manual titration with 1 M NaOH. After dialysis into a buffer containing 0.2 M Tris and 0.01 M EDTA (pH 8.6) the succinylated proteins were subjected to mild reduction in 0.2 M mercaptoethanol and alkylation with 0.22 M iodoacetamide (Fleischman *et al.*, 1962).

The [^{3}H]MNBDF affinity-labeled, succinylated, partially reduced, and alkylated proteins were then gel filtered to separate their H and L chains, using a colum of 2.5 × 150 cm of G-150 Sephadex developed at room temperature with 0.01 M Tris buffer (pH 8.2) (Lenard and Singer, 1966). The fractions containing the H and L chains were separately pooled, concentrated, and assayed for their radioactivity.

In order to obtain some information about the distribution of the affinity label within the labeled H and L chains, aliquots of these samples were then subjected to pronase digestion in 0.2 M ammonium bicarbonate (pH 8.0) for 20 hr at 40° using 50 μg of enzyme/ml. The digested samples were brought to dryness, dissolved in pyridine–acetate buffer, and chromatographed on a column of PA-35 resin in a pyridine–acetate pH gradient as described by Benson *et al.* (1966), and as used by Thorpe and Singer (1969). The radioactivity of the effluent fractions was monitored. Some of the fractions from the PA-35 chromatography were then gel filtered (to determine the average size of the labeled peptide fragments) on 0.9 × 100 cm columns of P-2 Bio-Gel in 1:1:1 phenol–acetic acid–water (Thorpe and Singer, 1969). The relation between molecular weight and effluent volume from this column was determined with several different tritiated compounds, [^{3}H]MNBDF-treated B chains of insulin (mol wt ~3700), [^{3}H]*m*-nitrobenzeneazotyrosylphenylalanine (mol wt 477), [^{3}H]*m*-nitrobenzeneazotyrosine (mol wt 330), and [^{3}H]*m*-nitroaniline (mol wt 138).

Radioactivity measurements were carried out in a 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation mixture in toluene, containing 33% Triton X-100 by volume. Counting efficiency was determined by the addition of internal standards of [^{3}H]toluene to the mixtures. A Beckman Model LS-200B instrument was used.

For the spectral assays, the nonradioactively affinity-labeled proteins were precipitated, washed, and analyzed, following the procedure of Wofsy *et al.* (1962). Alkaline difference spectra of the labeled whole proteins, but not of their separated chains, were obtained.

Equilibrium dialysis experiments were carried out at 4° in 0.1 M phosphate buffer (pH 7.4), containing 0.02% NaN_3. The cells used were 0.1-ml microdialysis cells (Chemical Rubber Co., Akron, Ohio). [^{3}H]Dnp-ε-aminocaproic acid was the ligand whose reversible binding to HPC-3 protein was measured. In each experiment, after equilibration for 21 hr, triplicate 0.010-ml aliquots were removed from each side of the cell and their radioactivity was counted in scintillation fluid (containing 8.0 g of butylphenylbiphenylyloxadiazole, 0.5 g of 2[4-biphenylyl]5-phenyloxazole, and 100 ml of BBS-3 Bio-Solv solubilizer (Beckman) in 1 l. of toluene) for 15 min to a precision of better than 0.6%. The recovery of radioactivity was about 92%. Control experiments without protein present were preformed. Calculation of the binding data was performed in the usual manner (Karush and Karush, 1971), assuming an extinction coefficient at 280 nm of 1.46 ml/mg and a molecular weight of 160,000 for HPC-3 protein.

The [^{3}H]Dnp-ε-aminocaproate at a specific activity of 26.2 Ci/mol was synthesized by the reaction of 150 μmol of [^{3}H]2,4-dinitrofluorobenzene (stated specific activity before dilution, 25.3 Ci/mmol, Amersham-Searle) and 150 μmol of ε-aminocaproic acid (Chemical Procurement Labs, Inc.) in 3.3 ml of 66% ethanol containing 0.3 M $NaHCO_3$. After 3-hr reaction at room temperature, the unreacted dinitrofluorbenzene was removed by ether extraction, the product was precipitated with concentrated HCl, and was recrystallized from 50% methanol, mp 135–136°. Over 97% of the radioactivity migrated as a single spot in thin-layer chromatography on silica gel in two different solvent systems benzene–pyridine–acetic acid (80:20:2, v/v) and benzene–methanol (95:5, v/v).

For an additional characterization of the HPC-3 protein, its capacity to bind and induce a color change in the dye DnpNS was determined by a previously described spectrophotometric procedure (Metzger *et al.*, 1963).

Results

The results of radioactive affinity-labeling experiments with HPC-3 and with a sample of pooled mouse anti-Dnp antibodies examined in parallel are given in Table I. In the case of the mouse antibodies there was about six times as much radioactivity bound by the unprotected as compared to the protected samples, similar to the degree of specific labeling obtained earlier (Thorpe and Singer, 1969). With HPC-3 protein, several independent experiments yielded a ratio of radioactivity bound by the unprotected and protected

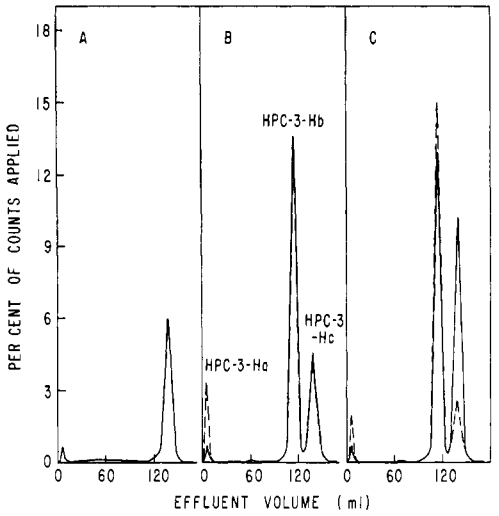

FIGURE 1: Radioactivity elution profiles from PA-35 chromatography of Pronase digests of affinity-labeled H chains: (A) from mouse anti-Dnp antibodies; (B and C) two independent experiments with HPC-3 myeloma protein. Full lines are for unprotected samples, dashed lines for protected. The protected H chain of the anti-Dnp antibodies was not studied.

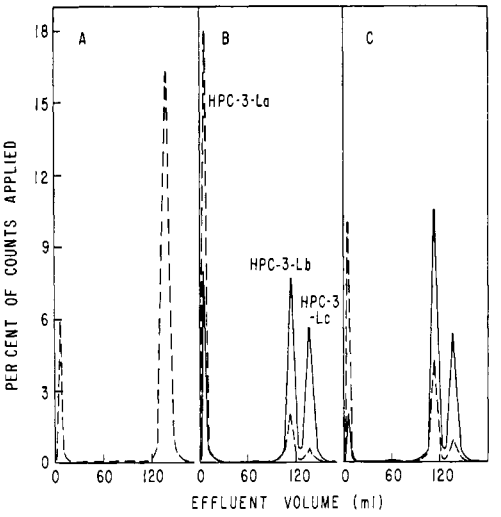

FIGURE 2: Radioactivity elution profiles from PA-35 chromatoggraphy of Pronase digests of affinity-labeled L chains: (A) from mouse anti-Dnp antibodies; (B and C) two independent experiments with HPC-3 myeloma proteins. These experiments were from the same labeled proteins as in Figure 1. Full lines are for unprotected samples, dashed lines for protected. The unprotected L-chain sample of the anti-Dnp antibodies was not available, but from other experiments (Thorpe and Singer, 1969) would be expected to give an elution profile similar to that shown for the protected sample.

samples of between two and three. Although the specificity of the reaction was therefore lower for HPC-3 than for the antibodies, it is nevertheless highly significant. The HPC-3 protein appears to be more nonspecifically reactive than the antibodies toward MNBDF, since the protected samples of the former protein were more highly labeled. Increasing the concentration of protector did not decrease the amount of nonspecific labeling of HPC-3.

The distribution of label between the H and L chains of the anti-Dnp antibodies in the one experiment in Table I was nearly equal on a molar basis. In previous experiment slightly more label (1.5:1 to 2:1) was found on the H than on the L chains (Thorpe and Singer, 1969). By contrast, with HPC-3 protein about ten times as much radioactive label was found on H compared to L chains.

The radioactively labeled H and L chains of unprotected and protected anti-Dnp antibodies and HPC-3 protein were digested with Pronase and chromatographed on a column of PA-35 resin in a pyridine–acetate pH gradient. From the radioactivity of the effluent fractions, the distribution of only the affinity-labeled peptide fragments was observed. Under the conditions used, this fractionation was highly reproducible. With affinity-labeled mouse anti-Dnp antibodies, Pronase digestion releases the label from both H (Figure 1A) and L (Figure 2A) chains predominantly as a single peak fraction eluting from the PA-35 column at the characteristic effluent volume of 137 ml. This peak is attributable to free *m*-nitrobenzeneazotyrosine (Thorpe and Singer, 1969; see below).

The labeled fragments from the Pronase-digested chains of HPC-3 protein were more complex, however. Consider the H chains first. In two separate experiments (Figure 1B,C), three radioactive fractions were recovered from the PA-35 chromatography; HPC-3-Ha eluting with the front, HPC-3-Hb eluting at 114 ml, and HPC-3-Hc eluting at 137-ml effluent volume. HPC-3-Hb was the major fragment. The ditribution of radioactive fragments was very similar for the unprotected and protected samples, as shown in Figure 1B,C where the ordinate is the percent of radioactivity applied to the column. (It should be remembered,however, that about three times as much *total* label was present on the unprotected as compared to the protected H chains, see Table I.) In order to characterize the average sizes of these fragments, they were each gel filtered on the calibrated P-2 Bio-Gel column (Figure 3). HPC-3-Ha eluted with the front, and was therefore of molecular weight greater than 2000. The elution volume of HPC-3-Hb corresponded to a molecular weight

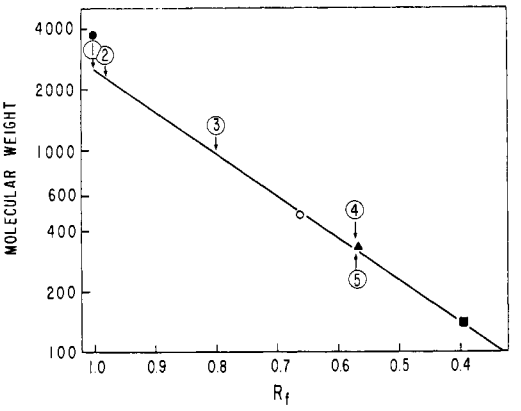

FIGURE 3: Sizing experiments with affinity-labeled fragments proproduced by Pronase digestion. Gel filtration was carried out on columns of P-2 Bio-Gel in 1:1:1 phenol–acetic acid–water. R_F is the ratio of the void volume of the column to the effluent volume of the peak fraction. The column was calibrated according to molecular weight using the four compounds: (●) [^{3}H]MNBDF-treated B chains of insulin; (○) [^{3}H]*m*-nitrobenzeneazotyrosylphenylalanine; (▲) [^{3}H]*m*-nitrobenzeneazotyrosine; (■) [^{3}H]*m*-nitroaniline. The affinity-labeled peptides eluted with R_F values indicated by the numbered arrows: ① HPC-3-La (protected); ② HPC-3-Ha (protected); ③ HPC-3-Hb (unprotected); ④ HPC-3-Hc (unprotected); and ⑤ the major labeled fragment from protected L chains of the anti-Dnp antibodies (Figure 2A). The designations of these fragments are as in Figures 1B and 2B, and in the text.

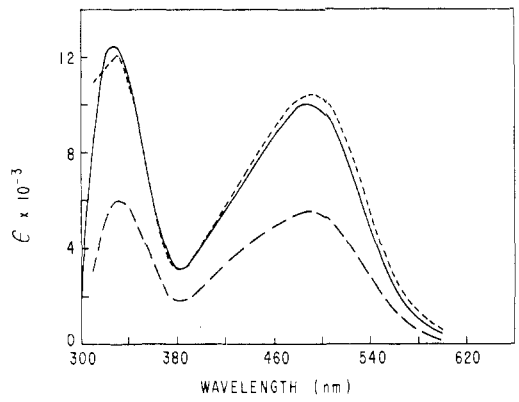

FIGURE 4: Difference spectra in 0.1 N NaOH of the affinity-labeled mouse anti-Dnp antibodies (large dashes); of the affinity-labeled HPC-3 protein (small dashes); and of the model compound *m*-nitrobenzeneazo(*N*-chloracetyl)tyrosine (full curve). In the case of the proteins, the extinction coefficients are calculated per mol of labeled protein. From these spectra it was calculated that the mouse anti-Dnp antibodies were labeled to the extent of 0.56 mol/mol of protein, and HPC-3 to 1.05 mol/mol of protein, in these experiments with nonradioactive MNBDF.

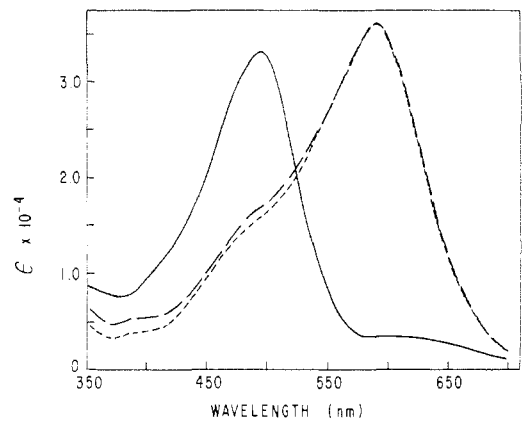

FIGURE 6: Spectra of DnpNS in free solution (small dashes); of a mixture containing 7.93×10^{-6} M DnpNS and 4.08×10^{-5} M HPC-3 protein (large dashes); and of a mixture containing 1.18×10^{-5} M DnpNS and 6.16×10^{-5} M mouse anti-Dnp antibodies (full curve); all in pH 7.40 phosphate buffer $\Gamma/2 = 0.2$.

of about 950, while HPC-3-Hc eluted at the same position as [^{3}H]*m*-nitrobenzeneazotyrosine, as expected.

The labeled fragments from the Pronase-digested L chains of HPC-3 protein also were separated into three similar fractions by chromatography on the PA-35 resin (Figure 2B,C). With the L chains, however, the labeled fragments from the unprotected and protected samples had quite different characteristics. The predominant labeled fragments (HPC-3-La, protected) recovered from the *protected* L chains eluted with the front from the PA-35 column, and also eluted with the front from the P-2 Bio-Gel column (Figure 3), indicating an average molecular weight for the fragments of greater than 2000. With the *unprotected* L-chain samples, however, fractions retarded on the PA-35 column constituted the largest proportion of the labeled fragments that were recovered. (Insufficient amounts of these latter fractions were available for P-2 Bio-Gel sizing experiments.) This indicates that the distribution of the affinity label within the L chains of HPC-3 protein was markedly different for the unprotected and protected samples.

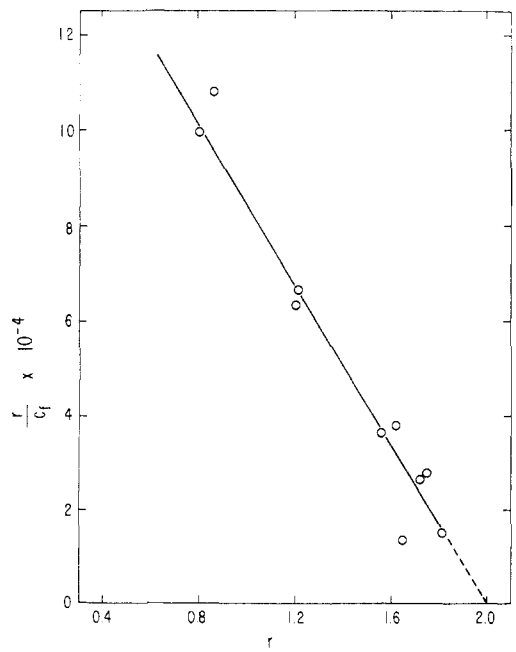

FIGURE 5: Equilibrium dialysis data for the binding of [^{3}H]Dnp-ε-aminocaproate to HPC-3 protein in 0.1 M phosphate buffer (pH 7.4) at 4°. See text for further details.

In separate experiments with nonradioactive MNBDF, the spectral properties of affinity-labeled HPC-3 protein and of mouse anti-Dnp antibodies were compared. As was demonstrated by Good *et al.* (1967), the difference spectra of the labeled proteins in alkali can readily distinguish between *m*-nitrobenzeneazotyrosine or *m*-nitrobenzeneazohistidine as the predominant product of the affinity-labeling reaction. The results given in Figure 4 show that the spectra of affinity-labeled HPC-3 protein and anti-Dnp antibodies are closely similar to one another and to the spectrum of the model compound, the *N*-chloracetyl derivative of *m*-nitrobenzeneazotyrosine.

The results of equilibrium dialysis experiments measuring the binding of [^{3}H]Dnp-ε-aminocaproate to HPC-3 protein are presented in Figure 5. The plot of r/C_f *vs.* r, where r is the ratio of the number of mol of hapten bound per mol of protein and C_f the concentration of free hapten, is a straight line which extrapolates to the value 2.0 for the number of binding sites per molecule of HPC-3 protein. The value of K_0, the association constant, is $8.4 \pm 0.2 \times 10^4$ M^{-1}. A Sips plot (*cf.* Karush and Karush, 1971) of the same data yields a value of the heterogeneity index $a = 1.0 \pm 0.1$.

Studies of the noncovalent binding of the dye DnpNS to HPC-3 protein were undertaken to further characterize the active sites of this nitrophenyl-binding protein. Upon binding to rabbit anti-Dnp antibodies at pH 7.4, DnpNS undergoes a marked color change due to the protonation of its naphtholate O^- group (Metzger *et al.*, 1963). The same profound spectral shift upon DnpNS binding to mouse anti-Dnp antibodies is shown in Figure 6. The addition of DnpNS to HPC-3 under the same conditions, however, produces no detectable spectral change (Figure 6). This absence of spectral change might either be due to an absence of binding of DnpNS to HPC-3 active sites at the concentration studied, or to binding that was not accompanied by the protonation of DnpNS. To decide between these alternatives, a competi-

tion experiment was performed (Figure 7). The spectrum of a mixture of DnpNS and mouse anti-Dnp antibodies was determined with and without an excess of HPC-3 protein added, care being taken to keep the two solutions otherwise identical. The spectrum with the HPC-3 protein present showed a small but significant shift from the protonated (absorbance maximum at 486 nm) to the unprotonated form (absorbance maximum at 590 nm). Control experiments under the same conditions but with normal mouse Ig (instead of HPC-3) added in excess to the solution containing anti-DNP antibodies and DnpNS produced no such spectral shift. This shift can only be explained as due to the dissociation of a small amount of the DnpNS that was bound to the anti-Dnp antibodies and its binding in the *unprotonated* form to the HPC-3 protein in the mixture of the dye and the two proteins. From the concentrations of the antibodies and HPC-3 protein in the mixture (assuming each protein to be pure and to have two active sites per molecule), and the extent of the spectral change upon the addition of HPC-3, we estimate that the ratio of the apparent equilibrium constants for the binding of DnpNS to mouse anti-Dnp antibodies and to HPC-3 protein is about 300.

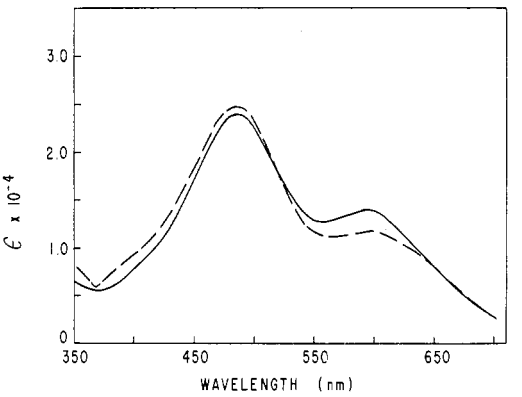

FIGURE 7: Spectra of two DnpNS-containing mixtures: (dashed line) 6.46×10^{-6} M mouse anti-Dnp antibodies and 7.04×10^{-6} M DnpNS; and (full curve) 6.46×10^{-6} M mouse anti-Dnp antibodies, 7.04×10^{-6} M DnpNS, and 3.23×10^{-5} M HPC-3 protein; all in pH 7.40 phosphate buffer, $\Gamma/2 = 0.2$.

Discussion

In the last several years, a considerable number of purified homogeneous myeloma proteins have been found to bind reversibly to specific small molecule ligands. The specificity and stoichiometry of this binding resembles that of antibodies elicited to these ligands, although in general the binding affinities of the myeloma proteins are smaller than the elicited antibodies. These ligand-binding myeloma proteins are of great interest for studies of the nature of antibody specificity and the structures of specific antibody active sites, particularly because they are homogeneous proteins whereas most elicited antibodies are heterogeneous. On the other hand, it has been unclear to what degree the structural details of the active sites of any ligand-binding myeloma protein are representative of the active sites of elicited antibodies in the same species.

The nitrophenyl-binding myeloma protein HPC-3 is an IgG2a mouse immunoglobulin, and is therefore of special interest because the majority of anti-Dnp antibodies elicited by hyperimmunization of mice with Dnp-antigens are of the IgG classes. On the other hand, most of the nitrophenyl-binding myeloma proteins induced in mice, including the one most extensively studied so far, MOPC-315, are IgA proteins.

The equilibrium dialysis studies recorded in Figure 5 show that HPC-3 protein has two sites per molecule capable of reversibly binding [^{3}H]Dnp-ε-aminocaproate. As expected of a homogeneous myeloma protein, a single value of K_0 characterizes the binding affinity; the plot of r/C *vs.* r is a straight line, and the Sips heterogeneity index $a = 1.0$. Warner and Ovary (1970) give a value of $K_0 = 8 \times 10^4$ M^{-1} for the binding of ε-Dnp-lysine to HPC-3 protein. The reversible binding properties of HPC-3 protein for Dnp ligands that have so far been discussed therefore are similar to those of elicited anti-Dnp antibodies.

Affinity labeling provides one means to probe the active sites of immunoglobulin molecules. In order for affinity labeling to occur with measurably large specificities, the structure of the reversible complex formed between the affinity-labeling reagent and the active site, as well as the nature and stereochemistry of certain amino acid residues in the active sites, have to be appropriate (Wofsy *et al.*, 1962). A large fraction (at least 50% (Good *et al.*, 1967)) of the active sites of *pooled* rabbit anti-Dnp antibodies, and a comparable fraction of pooled mouse anti-Dnp antibodies (Thorpe and Singer, 1969) are specifically affinity labeled with the reagent MNBDF at tyrosine residues on both the H and L chains. The results presented in this paper show that HPC-3 protein is also specifically affinity labeled by this reagent, by the criterion that two to three times as much label is attached to the unprotected protein compared to the protein protected by Dnp-aminocaproate (Table I). This difference between the labeling of unprotected and protected samples is highly significant; with one other nitrophenyl-binding mouse myeloma protein (S129, unpublished experiments of N. Martin, A. Sher, and S. J. Singer), the ratio of label on the unprotected and protected protein was 1.1 ± 0.1. Furthermore, spectral analyses (Figure 4) show that tyrosine residues are the exclusively labeled residues in the HPC-3 protein, as was found with rabbit and mouse anti-Dnp antibodies, and with the mouse myeloma protein MOPC-315 (Metzger and Potter, 1968).

With anti-Dnp antibodies from several species, the MNBDF label is distributed between the H and L chains in molar proportions between 1:1 and 5:1 (Good *et al.*, 1968). With HPC-3, however, there is ten times as much affinity label on H as on L chains. Whereas the amount of label on the unprotected H chains is clearly larger than on protected H chains, the total amount of label on the L chains is so small that it is difficult to be certain that the amount of label on the unprotected L chains is significantly larger than on the protected L chains (Table I). On the other hand, the labeled peptide fragments released by Pronase digestion are clearly different for the unprotected and protected L chains (Figure 2B,C). The major portion of the recovered labeled peptide fragments appears to be of large average size (mol wt >2000) from the protected L chain, whereas the major amount of label from the unprotected L chain appears to be distributed as free *m*-nitrobenzeneazotyrosine and small oligopeptide fragments. This can only mean that despite the not-too-different amounts of label on the two samples, the distribution of the label within the amino acid sequence of HPC-3 L chain must be different for the unprotected and protected samples, and therefore, that the label attached to the L chains of the unprotected sample must also be largely specific to the active sites of HPC-3 protein.

In their studies of the affinity labeling of MOPC-315 protein with [^{3}H]MNBDF, Metzger and Potter (1968) found that approximately 85% of the specific affinity label was attached to the L chains. No fragmentation studies were reported with the labeled H chains. In the light of the results reported in this paper, however, it would be interesting to determine whether the small amount of affinity label that does appear on the H chains of MOPC-315 is also specific to the active sites.

The problem of the ratio of affinity label that is bound to H and L chains has been discussed in some detail elsewhere (Singer and Doolittle, 1966; Singer *et al.*, 1971). The nearly equivalent amounts of label on the H and L chains of heterogeneous anti-Dnp and other anti-hapten antibodies have been interpreted to indicate that both H and L chains participate in the formation of *each* antibody active site (Singer and Doolittle, 1966; Singer and Thorpe, 1968). At first sight, however, it might be thought that the findings that the affinity label on HPC-3 protein is 90% on H chains, whereas with MOPC-315 protein the affinity label is 85% on L chains, are very disparate, and contradict the interpretation of the labeling results with the heterogeneous antibodies. Two factors must be appreciated, however, (1) the small amount (<10%) of label on the more lightly affinity-labeled chain of a myeloma protein may also be specific to the site; and (2) the ratio of label found on the two chains could vary quite significantly without much change in the structure of the active site. As was pointed out elsewhere (Singer *et al.*, 1971) the ratio of the amounts of two different affinity-labeled products i and j for a given population of homogeneous antibody active sites would be given by $\exp[(\Delta G_j^{\ddagger} - \Delta G_i^{\ddagger})/RT]$, where $\Delta G_i^{\ddagger}$ and $\Delta G_j^{\ddagger}$ are the free energies of activation for the covalent bond forming reactions between the reversibly bound affinity-labeling reagent with the groups i or j, respectively, in the active site. A ratio of 10 for the labeled products i and j would be expected if $\Delta G_j^{\ddagger} - \Delta G_i^{\ddagger}$ was only about 1.4 kcal/mol at 27°. In other words, relatively small differences in the free energies of activation for the affinity-labeling reaction with different groups on the H and L chains in a given active site could lead to apparently large changes in the ratio of labeling of the two chains. The affinity-labeling results obtained with HPC-3 and MOPC-315, even if we accept the proposition that these proteins are representative of anti-Dnp antibodies (see below, however), are not therefore necessarily inconsistent with the hypothesis that H and L chains both contribute significantly to the structure of each antibody active site.

Up to this point we have emphasized the similarities in the affinity-labeling characteristics of HPC-3 and mouse anti-Dnp antibodies. We now proceed beyond this point, and examine the question, how similar are the active sites of HPC-3 and other nitrophenyl-binding myeloma proteins to the active sites of elicited anti-Dnp antibodies? Several lines of evidence suggest that significant structural differences exist between the active sites of the majority of elicited antibody molecules and of the individual myeloma proteins so far studied. These are as follows. (1) Pronase digestion of affinity-labeled mouse anti-Dnp antibodies releases most of the specific label on the H chains as free *m*-nitrobenzeneazotyrosine (Figure 1A). Similar results are obtained for the specific label on the L chains (N. O. Thorpe and S. J. Singer, unpublished studies; only the result for protected L chains was available for Figure 2A). This means that the peptide bonds on both the amino- and carboxy-terminal sides of the labeled tyrosine residue(s) must be largely hydrolyzed by Pronase despite the fact that several different amino acid residues may occupy these neighboring positions in different H and L chains in the heterogeneous population (Thorpe and Singer, 1969). By contrast, however, Pronase digestion of affinity-labeled H and L chains of HPC-3 protein releases only a small fraction of the label as free *m*-nitrobenzeneazotyrosine, the major portion of the label from both chains being released as larger peptide fragments. This indicates that the peptide bonds from the labeled tyrosine residue(s) to the nearest-neighbor amino acid residues are generally not readily susceptible to Pronase digestion, and that these residues must therefore be different in the chains of HPC-3 from the chains of elicited anti-Dnp antibodies. The fact that HPC-3 protein was derived from the NZB mouse strain and the anti-Dnp antibodies from Swiss-Webster mice is not likely to be responsible for this profound difference. One possible explanation is that the tyrosine residue(s) which are affinity labeled in the HPC-3 protein are at specific positions in the amino acid sequence of the variable segments of the H and L chains which are different from the positions of the tyrosine residue(s) labeled on the majority of the antibody chains.

(2) The reversible binding of Dnp ligands to the active sites of the antibodies and myeloma proteins results in characteristically different optical properties. Differences are not evident in the *affinity* of these proteins for a ligand such as Dnp-lysine. With MOPC-315, for example, the K_0 for Dnp-lysine is 2×10^7 M^{-1} (Eisen *et al.*, 1968); with HPC-3, K_0 for Dnp-aminocaproate is 8.4×10^4 M^{-1} and is reported to be 8×10^4 M^{-1} for Dnp-lysine (Warner and Ovary, 1970). These values can be considered as falling within the range exhibited by elicited anti-Dnp antibodies. Characteristic differences appear, however, in the extrinsic Cotton effects exhibited upon binding Dnp-lysine to the myeloma proteins and the antibodies (Glaser and Singer, 1971). Although a ligand such as Dnp-lysine is optically inactive in the wavelength region corresponding to the Dnp absorption bands when it is in free solution, it becomes optically active upon binding to an asymmetric environment such as an antibody combining site. Despite the heterogeneity of pooled mouse anti-Dnp antibodies, the circular dichroism (CD) spectrum in the wavelength region of the Dnp absorption band which is generated upon binding Dnp-lysine to the antibodies, appears to be fairly characteristic (Glaser and Singer, 1971). In other words, the majority of the antibody active sites form Dnp complexes with quite similar CD optical characteristics. On the other hand, the CD spectra generated on binding Dnp-lysine to MOPC-315 (Glaser and Singer, 1971); Rockey *et al.*, 1971) and to HPC-3 (M. Glaser and S. J. Singer, to be published) are radically different from one another, and from those of the mouse antibody system. These spectra reflect the summation of interactions between the bound ligand and nearby amino acid residues in the active sites, and these results therefore suggest that the detailed structures of most of the active sites of mouse anti-Dnp antibodies are distinguished from those of the two myeloma proteins studied.

(3) The characteristics of the binding of the dye DnpNS to anti-Dnp antibodies and to the myeloma proteins are significantly different. The major fraction, if not all, of the active sites of heterogeneous rabbit and mouse anti-Dnp antibodies bind the *protonated* form of DnpNS preferentially at pH ~7 (Metzger *et al.*, 1963; Figure 6). That is, antibody-bound DnpNS has the pK of its naphthol ionization shifted to about 9.5, whereas it is about 6.5 in free solution. It has been suggested (Metzger *et al.*, 1963) that this reflects a characteristic hydrophobicity of regions within the active sites

of the antibody molecules. It is therefore of interest that the addition of DnpNS to MOPC-315 at pH ~7 produces no color change such as is characteristic of binding to antibodies (H. Metzger, personal communication). The results shown in Figures 6 and 7 indicate that DnpNS is indeed bound, if only weakly, to HPC-3 protein, but it is bound in the *ionized* rather than the protonated state at pH 7.4. These facts suggest that the active sites of these myeloma proteins are more hydrophilic than those of elicited antibodies.

Our conclusion is that careful evaluation by several criteria suggests that the *detailed structures* of the active sites of the nitrophenyl-binding myeloma proteins MOPC-315 and HPC-3 are significantly different from those of the major fraction of elicited mouse anti-Dnp antibodies. This conclusion is somewhat at odds with an increasing tendency to regard these myeloma proteins as closely representative of elicited antibodies which bind the same ligands. It introduces a cautionary note, which will no doubt be largely ignored, about the relevance of *detailed* chemical and physical studies of the active sites of myeloma proteins to the active sites of elicited antibodies. In another direction, it should raise further questions about the nature of elicited antibodies, and why they appear, at least in some cases, to have active sites that are very highly selected upon primary immunization with the antigen. For example, as has been extensively studied by Little, Eisen, and their coworkers (*cf.* Eisen *et al.*, 1969), antibodies elicited by Dnp-antigens and by Tnp-antigens are chemically and physically readily distinguishable from one another despite the extensive cross-reactions *in vitro* of Dnp- and Tnp-haptens. These results strikingly demonstrate that more than just binding affinity for a hapten is involved in the selection and stimulation of an anti-hapten–antibody response.

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CORRECTION

On page 4853 of the December 5 (No. 25), 1972, issue of *Biochemistry*, the title was inadvertently omitted from the paper by David B. Finkelstein, John Blamire, and Julius Marmur. The following title should appear: "Isolation and Fractionation of Yeast Nucleic Acids. II. Rapid Isolation of Mitochondrial Deoxyribonucleic Acid by Poly(L-lysine) Kieselguhr Chromatography."