Recovery of Binding Activity in Reconstituted Mouse Myeloma Proteins*

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ABSTRACT: Requirements for the formation of immunoglobulin active sites have been investigated with mixtures of heavy and light chains derived from several mouse myeloma proteins, including A-myeloma proteins that bind 2,4-dinitrophenyl (DNP) ligands. Full binding activity was recovered in recombinant molecules in which both heavy and light chains were derived from the same parent molecule. Very little or no activity was demonstrated in recombinants in which one chain was derived from a protein with binding activity and the alternate chain, from a nonbinding protein, or in recombinants where heavy and light chains were derived from two different myeloma proteins with anti-DNP activity. The results suggest that unique pairs of heavy and light chains are required to form a particular combining site.

In early structural studies of immunoglobulins, it was shown that the component heavy (H) and light (L) chains could be separated in dissociating solvents after the reduction and alkylation of the interchain disulfide bonds (Fleischman *et al.*, 1962). Four-chain molecules could be re-formed from mixtures of H and L chains from antibodies (Fougereau *et al.*, 1964; Hong and Nisonoff, 1966; Roholt *et al.*, 1965, 1967), nonspecific immunoglobulins (Olins and Edelman, 1964), or myeloma proteins (Grey and Mannik, 1965) after dialysis against neutral aqueous buffers. The recombined molecules were stable under nondissociating conditions and resembled native immunoglobulins in physical and antigenic properties. With antibodies, however, full ligand binding activity was not recovered.

The availability of mouse myeloma proteins with antibody activity has allowed the characterization of the binding properties of homogeneous immunoglobulins after polypeptide chain separation and recombination. We present here the results of studies undertaken with two mouse myeloma proteins, 315 and 460, which bind 2,4-dinitrophenyl (DNP)[1] ligands and two myeloma proteins, 173 and 176, which lack DNP specificity. We have examined the recovery of binding activity and of normal immunoglobulin structure in recombinant molecules derived from homologous and heterologous[2] chain mixtures (Bridges and Little, 1970).

Materials and Methods

Plasmacytomas and Protein Purity. Plasma cell tumors MOPC-173, 315, and 460 were obtained from M. Potter, at the National Cancer Institute, and S-176 from M. Weigert, The Salk Institute, and maintained by serial transplantation as subcutaneous solid tumors in Balb/c mice. A-myeloma proteins 315 and 460 were purified from the pooled serum of tumor-bearing mice by the method of Jaffe *et al.* (1971); A-myeloma protein 176, by the method of Eisen *et al.* (1968); G-myeloma protein 173, by the method of Potter (1967).

Immunoelectrophoresis was performed on each purified myeloma protein preparation at 20–30, 10, and 2–3 mg per ml; the most concentrated sample was then subjected to microzone electrophoresis on cellulose acetate strips (Beckman) which were subsequently stained with ponceau red, cleared, and scanned in a Beckman Analytrol. By immunoelectrophoresis, the preparations were shown to be contaminated by one or more additional proteins when tested at 20–30 mg/ml; however, only the specific myeloma protein was demonstrated in the 2–3-mg/ml samples, except in the case of protein 176 where a diffuse second component was noted. Microzone analysis detected no contaminants in samples of protein 460 and 315, and a small anodal shoulder in the case of protein 173. The highest concentration of protein 176 tested in this manner was 14 mg/ml; only the myeloma protein was observed.

Fluorescence quenching measurements (Velick *et al.*, 1960) were used as an additional criterion of purity for proteins 315 and 460. At 4°, the initial fluorescence of all preparations of protein 315 was quenched 60–65% by ε-DNP-L-lysine; of all preparations of protein 460, 33–35% by dinitronaphthol at total ligand concentrations of 8×10^{-7} M. These values agree with those obtained by others for purified proteins 315 and 460 (Eisen *et al.*, 1968; Jaffe *et al.*, 1971).

H- and L-Chain Separation. H and L chains were prepared from the purified myeloma proteins as outlined below. Reduction and alkylation was performed at 22–23° in the dark. Myeloma protein solutions (20–30 mg/ml), previously dialyzed against 0.15 M Tris-Cl (pH 8.0), 0.15 M NaCl, and 0.002 M Na_2EDTA, were reduced with 0.01 M dithiothreitol for 2 hr. Each preparation was then alkylated with 0.022 M iodoacetic acid for 15 min. The concentration of reducing and alkylating agents was then decreased by dialysis against

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[1] Abbreviations: DNP, the 2,4-dinitrophenyl group; ε-DNP-L-lysine, ε-2,4-dinitrophenyl-L-lysine; dinitronaphthol, 2,4-dinitro-1-naphthol; phosphate-buffered saline, 0.02 M potassium phosphate (pH 7.4)–0.15 M NaCl.

[2] Homologous refers to mixtures composed of H and L chains derived from the same immunoglobulin; heterologous, from two different immunoglobulins.

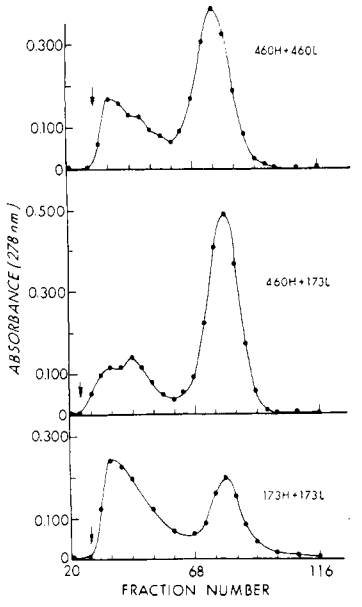

FIGURE 1: Separation of recombinant species from the other products of the chain mixtures by gel filtration on Sephadex G-150 in phosphate-buffered saline–0.002 M Na_2EDTA. Two columns (2.5 × 80 cm each) connected in tandem were used. The arrow marks the void volume, determined by Blue Dextran. The heterogeneous first peak was composed of aggregates containing H and L chains; the second peak was the recombinant species. In some experiments, free L chains were detected as a third peak, well separated from the preceding two. Human serum albumin, when present, eluted at a position overlapping extensively with L chains.

phosphate-buffered saline for several hours at 22–23° before the samples were applied to a Sephadex G-100 column (2.5 × 100 cm), at 4°, in 4.5 M urea–1 M propionic acid. Separate pools were made of the H and L chains, and mixtures made as described below.

Because of the method of their purification, this procedure constituted a second reduction and alkylation for proteins 460 and 315, without which quantitative yields of H and L chains were not obtained. These proteins were alkylated with ^{14}C- or ^{3}H-labeled iodoacetic acid during the purification, and with nonradioactive iodoacetic acid in the second alkylation. Proteins 173 and 176 were radioactively labeled during the one reduction and alkylation to which they were subjected.

Chain Recombination. H and L chains were mixed in the urea–propionic acid solvent or in 1 M propionic acid at an H/L absorbancy ratio (278 nm) of 2–3/1., and diluted to a final protein concentration of 300 μg/ml. Human serum albumin (Pentex, Kankakee, Ill.) was added to 500 μg/ml in some experiments as protection against chain denaturation. The chains were recombined by dialysis at 4° in three stages, each 16–24 hr in duration. The first dialysis was against a volume of deionized water sufficient to reduce the propionic acid concentration to 0.01 M; the second dialysis was against fresh deionized water, to 0.001 M propionic acid; and the third dialysis was against 0.01 M Tris-Cl (pH 8.0) of a volume sufficient to reach equilibrium with this buffer. The mixtures were then concentrated by lyophilization or ultrafiltration (Amicon Corp., Lexington, Mass.) in preparation for separation of the products of the recombination by gel filtration on Sephadex G-150 in phosphate-buffered saline–0.002 M Na_2EDTA.

Molar H/L Chain Ratio. The molar H to L chain ratio was determined from recombinant material in which one chain (H or L) was labeled with [^{14}C]iodoacetic acid and the alternate chain, with [^{3}H]iodoacetic acid. The $^{14}C/^{3}H$ ratio that corresponded to a 1:1 molar ratio of H to L was taken to be the ratio of the specific activity of the H-chain preparation (cpm/1.0 absorbance unit, 278 nm) to the specific activity of the L-chain preparation multiplied by the ratio of the total absorbance in the H-chain peak to the total absorbance in the L-chain peak in the initial chain separation.

Samples were precipitated with cold 5% trichloroacetic acid in preparation for counting. After a minimum of 2 hr at 4°, the precipitates were collected on filters (Millipore, 0.8 μ), washed with cold 5% trichloroacetic acid, followed by cold 0.1 N HCl. The filters were dried and the relative amounts of each isotope were determined in a toluene-based scintillation fluid by a Packard liquid scintillation counter.

Binding Measurements. Equilibrium dialysis was performed in small plastic chambers with 50 μl of protein separated by dialysis casing from 50 μl of [^{3}H]-ε-DNP-L-lysine at various concentrations. The chambers were rotated on a wheel at approximately 5 rpm at 4° for 24 hr. However, in experiments involving protein 315 chains, the chambers stood for 36 hr without agitation since this protein may be unusually susceptible to denaturation (Underdown *et al.*, 1970). At equilibrium, radioactivity measurements were made in Bray's solution (1960) by sampling from both sides of the chamber. Fluorescence quenching (Eisen, 1964) and difference spectra between bound and free DNP ligand (Little and Eisen, 1967) were performed as described previously.

Other Materials. ε-DNP-L-lysine (Sigma Chem. Co.), dinitronaphthol (Eastman Organic Chem.), dithiothreitol (P-L Biochemicals), [^{14}C]iodoacetic acid (7.6–13.4 mCi/mmole), and [^{3}H]iodoacetic acid (73.9 mCi/mmole) (New England Nuclear) were used as supplied by the manufacturer. Nonradioactive iodoacetic acid was recrystallized twice from diethyl ether–ligroin (bp 66–75°, Fischer Scientific Co.). [^{3}H]-ε-DNP-L-lysine was synthesized as described by Eisen *et al.* (1968), and the purity was established by thin-layer silica gel chromatography in water-saturated methyl ethyl ketone, by comparison of the absorbance spectrum to that of authentic ε-DNP-L-lysine, and by equilibrium dialysis with a purified rabbit antibody specific for the trinitrophenyl group, in which case ligand absorbance (360 nm) and radioactivity equilibrated indistinguishably. Urea was deionized prior to use by stirring overnight at 4° with a mixed-bed resin (Amberlite MB-3, Mallinckrodt).

Results

Efficiency of Recombination. Representative Sephadex G-150 elution profiles of protein 460 and 173 chain mixtures[3] and a 460 + 173 heterologous mixture are given in Figure 1. As is demonstrated there and in Table I, the yield of recombinant (material eluting at the position of the reduced-alkylated parent protein) was variable, depending on the particular combination of chains. Combinations in which one or both

[3] The chain mixture is designated by the number of the protein followed by the chain type, H or L; 460H + 460L means a mixture of homologous chains from protein 460. The recombinant species isolated from such a mixture is referred to as 460H–460L; the reduced-alkylated immunoglobulin which has been carried through the procedure up to, but not including, chain separation is designated, for example, 460 RA.

TABLE I: Efficiency of Recombination.

Mixture	Percentage A (278 nm) in Recombinant Peak[a]
460H + 460L	73
315H + 315L	41
173H + 173L	29
460H + 173L	69
173H + 460L	65
315H + 173L	19, 67[b]
173H + 315L	9, 40[b]
315H + 176L	72[b]
176H + 315L	66[b]
460H + 315L	73[b]
315H + 460L	74[b]

[a] Absorbance in recombinant peak as related to total absorbance recovered from Sephadex G-150 gel filtration. [b] Recombination in the presence of human serum albumin; absorbance related to total absorbance recovered in aggregate and recombinant peaks, since human serum albumin and free L-chain elution positions were coincident.

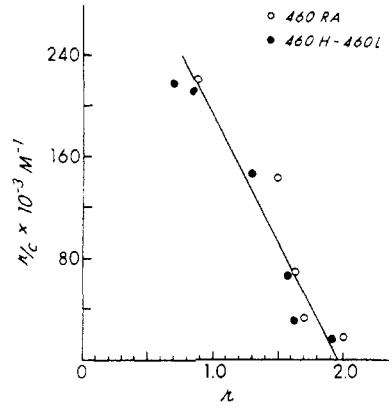

FIGURE 2: Binding of ε-DNP-L-lysine by 460 RA and 460H–460L, as determined by equilibrium dialysis. 50 μl of protein, 6.0×10^{-6} and 5.9×10^{-6} M for 460 RA and 460H–460L, respectively, was dialyzed against 50 μl of [^{3}H]-ε-DNP-L-lysine, specific activity 3.53×10^5 cpm/mμmole, at 4°. The buffer was phosphate-saline. Experimental points are plotted around the line of regression; the coefficient of correlation is −0.972. The K for ε-DNP-L-lysine, as determined from this plot is 1.9×10^5 M^{-1}. r is moles of hapten bound per mole of protein (150,000 molecular weight) at a free hapten concentration c.

chains were derived from protein 460 gave 65–70% of the material in the recombinant peak, whereas combinations in which one or both chains were derived from protein 315 (in the absence of albumin protection) gave approximately 10–40% of the material at that position. There was no correlation between the kind of mixture—homologous or heterologous—and the efficiency of recombination; rather, certain chain combinations resulted in poor H–L-chain interaction and aggregation was favored.

Homologous Chain Mixtures. In the extensive study of the DNP system, several binding assays have been developed for measuring different properties of the antibody and ligand. This is of particular importance for this study since one of the central aims was to determine how closely the combining site of the reconstituted protein resembled the active site of the native protein. The binding properties of the recombinants were therefore studied by equilibrium dialysis, by protein fluorescence quenching, and by ligand spectral shift.

The binding activity of the 460 homologous recombinant determined by equilibrium dialysis is shown in Figure 2 along with the data obtained with the reduced-alkylated parent 460 protein in the same experiment. The line of regression was calculated from the two sets of points; the coefficient of correlation was −0.972. The association constant, K, for [^{3}H]-ε-DNP-L-lysine, determined from the plot is 1.9×10^5 M^{-1}, and the line extrapolates to 2 binding sites per molecule. Corresponding data, not illustrated here, was obtained with the 315 protein. With the homologous recombinant of 315 chains, a K of 4.9×10^6 M^{-1} was observed; with the reduced-alkylated parent, a K of 5.6×10^6 M^{-1}. These values are within experimental error of that found previously (Eisen *et al.*, 1968). The data points were linear when plotted on Scatchard coordinates and extrapolation to the abscissa indicated 1.7 and 1.8 sites per molecule, respectively.

Figure 3 gives an example of the fluorescence quenching properties of the 315 and 460 homologous recombinants, their reduced and alkylated parent proteins, and other components of the recombination mixture. The fluorescence of the 315 recombinant was quenched by ε-DNP-L-lysine to approximately the same extent (60%) as the reduced-alkylated 315 protein. The 460 recombinant and the reduced-alkylated protein 460 gave identical fluorescence quenching curves with dinitronaphthol. In both cases (Figure 3), the fluorescence of the aggregated heavy and light chains was quenched 22%, and the H- and L-chain fluorescence, less than 10%, by the appropriate ligand.

To test the possibility that the fluorescence quenching obtained with the homologous recombinants could be due to

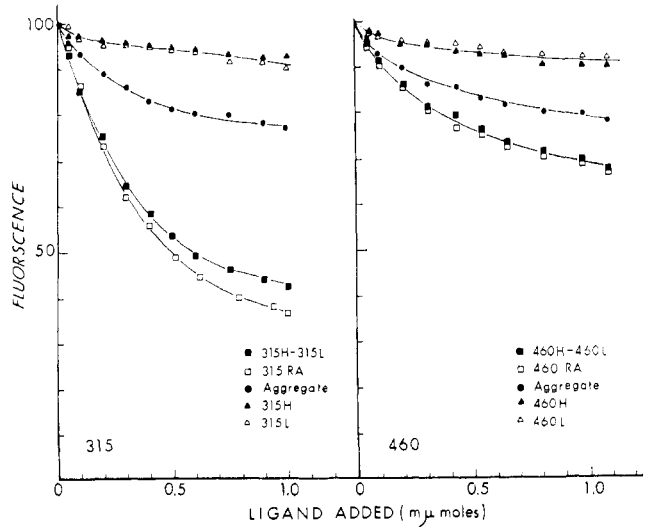

FIGURE 3: Fluorescence quenching, homologous chain mixtures. The fluorescence of the recombinant species, aggregate material, isolated H and L chains, and reduced-alkylated parent proteins was titrated with 5.0×10^{-6} M ε-DNP-L-lysine (protein 315) or 5.5×10^{-6} M dinitronaphthol (protein 460). All measurements were made at 4° in phosphate-buffered saline, with protein concentrations ranging from 35 to 55 μg per ml. Excitation was at 280 nm; fluorescence emission was recorded at 350 nm, in an Aminco-Bowman spectrofluorometer.

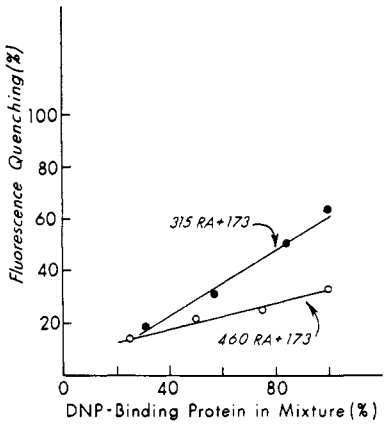

FIGURE 4: Relationship of the fluorescence quenched to the relative proportion of DNP-binding protein in a mixture with a nonbinding species. 315 RA or 460 RA was mixed in various proportions with the nonbinding protein 173; total protein concentrations ranged from 40 to 60 μg per ml. Each mixture was titrated with the appropriate ligand, ϵ-DNP-L-lysine (4.8×10^{-6} M) or dinitronaphthol (5.0×10^{-6} M), respectively. Each point represents the fluorescence quenched after approximately 1 mμmole of ligand had been added to 0.26–0.40 mμmole of the protein mixtures. All titrations were carried out at 4° in phosphate-buffered saline.

the contamination of an inactive recombinant population by unseparated parent molecules, the DNP-binding proteins were mixed in various proportions with the nonbinding protein 173. The DNP-binding protein was analogous to the unseparated parent population; the nonbinding protein, to a potentially inactive recombinant population.

As can be seen in Figure 4, a linear relationship existed between the fluorescence quenched and the percentage of DNP-binding protein present in the mixture. If the binding reported for the 315 and 460 recombinants were due to unseparated parent molecules, the proportion of these molecules would have had to approach 100%. Very much less than this degree of contamination would have been detected in the molar chain ratios of the recombinant species.

The difference spectrum between bound and free dinitronaphthol obtained with the 460 homologous recombinant and the reduced-alkylated 460 protein is shown in Figure 5. The maxima, at 478 and 405 nm, were coincident. The increase in molar absorptivity, $\Delta\epsilon_M$ (478 nm), of bound dinitronaphthol at 4° was 6100 for the recombinant and 6760 for the parent protein. Similar spectra, not shown here, were obtained

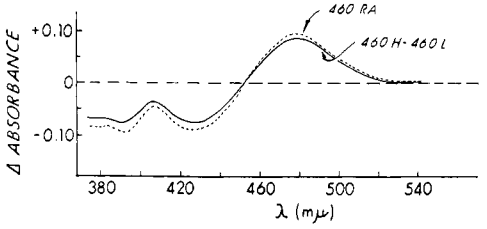

FIGURE 5: Difference spectra between free and bound dinitronaphthol with protein 460 RA and its homologous recombinant, 460H–460L. Spectra were obtained as the difference between protein plus ligand in the experimental cell and the same total concentration of each in separate sides of the tandem reference cell. The total ligand concentration was 2.4×10^{-5} M; the final protein concentrations, 6.0×10^{-6} and 5.9×10^{-6} M, for 460 RA and 460H–460L, respectively. Both spectra were determined at 4° in phosphate-buffered saline.

TABLE II: Recovery of Structure and Activity.

Recombinant	V_e/V_0[a]	$s_{20,w}$ (S)[b]	Molar H/L[c] Chain Ratio	K, M^{-1}, [^{3}H]-ϵ-DNP-L-Lys Equilibrium Dialysis
460H–460L	1.39	6.4	1.2	1.9×10^5
315H–315L	1.34	6.5	0.92, 0.98	4.9×10^6
173H–173L	1.40	6.2	0.73, 0.82	Not tested
460H–173L	1.39	6.1	1.1	$<3.0 \times 10^3$
173H–460L	1.44	6.5	0.8	$<1.0 \times 10^3$
315H–173L	1.32	6.0	0.8, 1.2, 1.0	$<1.0 \times 10^3$
173H–315L	1.38	6.6	1.3, 1.5	Not tested
315H–176L	1.36	6.6	1.5	$<3.0 \times 10^3$
176H–315L	1.37	6.6	1.8	$<1.0 \times 10^4$
460H–315L	1.40	6.0	2.0	1.5×10^5
315H–460L	1.38	6.0	1.5	$<1.0 \times 10^3$
Parent Proteins				
460 RA		6.6		1.9×10^5
315 RA	1.38	6.5		5.6×10^6
173 RA	1.43	6.3		Not tested

[a] The ratio of the elution position of the recombinant or reduced-alkylated parent protein to the void volume of the column, Sephadex G-150 in phosphate-buffered saline–EDTA. [b] Corrected sedimentation coefficient, determined in a Beckman analytical ultracentrifuge equipped with ultraviolet scanner. Conditions: speed 52,000 rpm, temperature, 22–25° in various experiments; solvent, 0.1 M NaCl–0.005 M potassium phosphate (pH 7.4). Protein concentration was approximately 1 mg/ml. [c] Determined from the ratio of ^{14}C and ^{3}H in the double-labeled recombinant material and the specific activity of the isolated chain preparations, as detailed in the text.

with the 315 homologous recombinant and its parent protein. $\Delta\epsilon_M$ (470 nm) for bound ϵ-DNP-L-lysine at 4° was 1150 with the recombinant, and 1400 with the reduced-alkylated 315 protein.

Heterologous Chain Mixtures. Mixtures of two kinds were studied: (a) those in which one chain was derived from a protein with anti-DNP activity, 315 or 460, and the alternate chain from a nonbinding protein, 176 or 173; and (b) mixtures in which one chain was derived from each of the two different immunoglobulins with anti-DNP activity. The particular combinations examined are listed in Table II. The sedimentation coefficients and gel filtration properties determined for these recombinants were comparable to those of the homologous recombinants and the reduced-alkylated parent proteins. The molar H/L ratios for the recombinants from 315H + 173L and 460 + 173 mixtures ranged from 0.8 to 1.2, and for the 315 + 460 and 315 + 176 mixtures, from 1.5 to 2.0.

No significant activity was recovered from most of these recombinants, as judged by fluorescence quenching and equilibrium dialysis. Table II summarizes the binding data obtained by the latter method. With the 460H–315L recombinant, the K value for [^{3}H]-ϵ-DNP-L-lysine was 3% of the value determined for the reduced-alkylated 315 protein. That this small amount of binding was characteristic of the protein from which the light chain was derived was shown by

the failure of dinitronaphthol, added at one-half the molar concentration of radioactive ligand, to inhibit the binding of [^{3}H]-ε-DNP-L-lysine. Protein 460 binds dinitronaphthol approximately 20 times better than ε-DNP-L-lysine, whereas protein 315 binds the latter ligand preferentially (Jaffe *et al.*, 1971). Had the specificity been characteristic of the heavy chain, it could have been argued that the binding was due to the contamination of the H-chain preparation by unseparated 460 parent molecules.

Discussion

Recovery of Structure. The extent to which H and L chains regain their native structure in a recombinant molecule can be evaluated by a number of criteria, some of which are more gross than others. For example, gel filtration position, sedimentation coefficient, and antigenic character (with the exception of recognition of idiotypic determinants) are weak criteria. We have shown here that recombinants resembling the reduced-alkylated parent molecule by the former two criteria failed to recover the precise molar chain composition of the native protein. Also, a homologous recombinant prepared from chains of protein 173 (not reported here) gave a reaction of identity in gel diffusion with the native protein and with the aggregated product of the chain recombination, when tested with a rabbit antiserum to Balb/c whole serum. Yet the aggregate was clearly different from the native molecule in size and polypeptide composition. Therefore, the information obtained by immunodiffusion with this kind of antiserum only serves to show that both chains of the parent protein were present.

The recovery of binding activity, as judged by equilibrium dialysis, is a more rigorous test of the recovery of structure. With nonbinding species, however, the molar ratio of H/L chains appeared to be the most significant criterion. In these studies, molar ratios of 0.8–1.2 were considered acceptable. The 460 homologous recombinant had a ratio of 1.2 and was by every activity measurement indistinguishable from the reduced-alkylated parent molecule; recombinant 315H–173L in three different experiments had ratios of 0.8, 1.2, and 1.0.

Several experiments yielded recombinants with molar chain ratios greater than 1.2 (see Table II). Some possible explanations are as follows. (a) Each of these mixtures contained a chain derived from protein 315. Since the light chain of this protein is believed to belong to an infrequently occurring type (Goetzl and Metzger, 1970; Schulenburg *et al.*, 1971), the L chain and its complementing H chain may be so different in structure as to make relatively poor interactions with most other chains. (b) The presence of albumin during the recombination may have interfered with chain–chain interaction, resulting in a less than normal L-chain complement. This seems unlikely because a mixture of 315H and 173L chains gave molar ratios of 0.8 and 1.2 in the absence of albumin, and 1.0 in its presence. (c) The calculation of the chain ratios may have been based on an incorrect assumption; the ratio of H/L absorbancies determined for the parent immunoglobulin might not, in some cases, have been appropriate for mixed molecules. However, if a particular heterologous mixture gave a high ratio, the reciprocal mixture might be expected to give a low ratio. This was not observed. (d) There may have been unequal labeling of the H- or L-chain preparations, resulting in denaturation of a fraction of the chains. The specific activity determined from the isolated H- and L-chain preparations then would not be an accurate reflection of the chains participating in the recombination process. By itself, this seems an unlikely explanation, since the chain ratios determined from the absorbancy of the H- and L-chain pools (data not reported here) of the reseparated recombinants were high also.

The importance of the problem of inappropriate chain ratios is difficult to assess. Olins and Edelman (1964) reported values of from 0.8 to 1.8 for human IgG recombinants. Other workers have simply stated that their ratios were near unity. For the homologous recombinants and several heterologous mixtures, the chain ratios observed were certainly acceptable. For chain combinations which were not favorable, the accumulation of several of the above explanations could have resulted in poor ratios.

Recovery of Activity. By all three criteria of binding, proteins 315 and 460 have been reconstituted to molecules with ligand binding properties of the reduced and alkylated parent molecule. It appears, therefore, that the proper noncovalent association of the chains for maximal binding affinity can be attained outside of an intact cell. This has also been shown by Klinman (1970) in experiments with conventionally induced anti-DNP antibodies. Monofocal antibodies, the homogeneous products of individual splenic foci, were reconstituted to full activity and homogeneity after the separation and recombination of their heavy and light chains.

Furthermore, there is no requirement for participation by antigen, as full recovery was demonstrated in its absence. Metzger and Mannik (1964) found that H and L chains of conventionally induced antibodies reassembled in the presence of hapten yielded recombinants with enhanced activity when compared to chains reassembled in its absence, possibly due to a directing influence of hapten on the conformation of the chains. The level of contamination by hapten or antigen of the immunoglobulin preparations used in this study was at maximum 4%, judged by the ratio of total absorbance at 360 nm to that at 278 nm. Considering the low affinity of the isolated chains, it seems unlikely that even this amount of contamination could have survived dissociation during chain separation or the dialysis step of the recombination procedure.

Eight heterologous chain combinations were studied. In no case was significant activity recovered. This result clearly suggests that full recovery of specific binding activity is a function, at least in these anti-DNP proteins, that requires participation by both chains of a particular pair. Grey and Mannik (1965) have shown under certain experimental conditions that if two myeloma L chains, one homologous and one heterologous, compete for a given H chain, preferential recombination of the homologous chain pair will occur. This would suggest that the interaction between the two chains of a given pair is better than the average random chain interaction and that this may be an important factor associated with the potential for activity in the corresponding antibody system. However, if the explanation for preferential recombination proves to be related to the previous association of the chains, then the dominant requirement for the selection of a chain pair as antibody must be the formation of an effective binding site, with the general affinity displayed by the members of a random chain pair sufficient for the stability of a four-chain molecule.

The extent to which nonhomologous H–L-chain pairs can form a recombinant molecule with an effective combining site is still not clear. Experiments with heterogeneous antibody

populations have shown that H and L chains can reassociate into molecules with some of the properties of the parent immunoglobulin population, but which are characterized by a reduced binding activity. This residual activity could be due to (a) nonhomologous chain pairing, in which case the association of chains within the antibody population to form effective active sites would be relatively permissive; or (b) predominantly to the re-formation of original chain pairs, which alternatively suggests that the association of two *particular* chains is required. Mixtures of proteins 315 and 460, potential members of a heterogeneous anti-DNP population, fail to display either recovery of full binding activity or normal four-chain structure. This emphasizes that ligand binding activity depends upon effective chain–chain interactions even when the nonhomologous chains are derived from molecules with similar combining sites.

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References

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Bridges, S. H., and Little, J. R. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 773.
Eisen, H. N. (1964), *Methods Med. Res. 10*, 106.
Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7*, 4126.
Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys. Supp. 1*, 174.
Fougereau, M., Olins, D. E., and Edelman, G. M. (1964), *J. Exp. Med. 120*, 349.
Goetzl, E. J., and Metzger, H. (1970), *Biochemistry 9*, 3682.
Grey, H., and Mannik, M. (1965), *J. Exp. Med. 122*, 619.
Hong, R., and Nisonoff, A. (1966), *J. Immunol. 96*, 622.
Jaffe, B. M., Simms, E. S., and Eisen, H. N. (1971), *Biochemistry 10*, 1693.
Klinman, N. R. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 571.
Little, J. R., and Eisen, H. N. (1967), *Biochemistry 6*, 3119.
Metzger, H., and Mannik, M. (1964), *J. Exp. Med. 120*, 765.
Olins, D. E., and Edelman, G. M. (1964), *J. Exp. Med. 119*, 789.
Potter, M. (1967), *Methods Cancer Res. 2*, 105.
Roholt, O., Radzimski, G., and Pressman, D. (1965), *J. Exp. Med. 122*, 785.
Roholt, O., Radzimski, G., and Pressman, D. (1967), *J. Exp. Med. 125*, 191.
Schulenburg, E., Lynch, R., Simms, E., Bradshaw, R., and Eisen, H. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 468.
Underdown, B. J., Simms, E. S., and Eisen, H. N. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 437.
Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1470.

Metabolism of Benzoic Acid by Bacteria. Accumulation of (−)-3,5-Cyclohexadiene-1,2-diol-1-carboxylic Acid by a Mutant Strain of *Alcaligenes eutrophus**

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ABSTRACT: A mutant strain of *Alcaligenes eutrophus* blocked in benzoic acid catabolism converts benzoic acid into a previously unknown compound which was identified as 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. The incorporation of two atoms of molecular oxygen was established by mass spectroscopic examination of the pattern of incorporation of ^{18}O from an $^{18}O_2$-enriched atmosphere during formation of the compound from benzoic acid by whole cells. This evidence, together with that favoring the cis configuration for the compound, suggests that it is formed by reduction of a peroxide produced from benzoic acid in a dioxygenase-mediated reaction.

Several monosubstituted benzoic acids are also converted into the corresponding substituted 3,5-cyclohexadiene-1,2-diol-1-carboxylic acids by benzoate-induced cell suspensions, indication that the enzyme(s) responsible for the conversion are relatively nonspecific. A scheme is presented which includes this previously unknown compound as an intermediate in the conversion of benzoic acid into catechol by bacteria.

The oxidation of benzoic acid by bacteria has been known for many years to proceed *via* catechol (Stanier, 1948). Molecular oxygen is known to participate, but little mechanistic detail has been learned about the conversion of benzoic acid into catechol, in part because it has been difficult to demonstrate appreciable oxidation of benzoic acid in cell-free extracts.

Because of the chemical complexity of the process it has been suggested that several enzymatic steps are involved in the

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