Resonance Raman Spectroscopic Studies of 2,4-Dinitrophenyl Hapten–Antibody Interactions†

Paul R. Carey,* Arnold Froese, and Henry Schneider

ABSTRACT: Resonance Raman spectra have been obtained of 2,4-dinitrophenyl haptens free in solution and complexed with homologous, specifically purified rabbit antibodies. The haptens were ε-2,4-dinitrophenyl-L-lysine and two azo derivatives, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalenedisulfonic acid (A-Dnp) and 1-hydroxy-2-(2,4-dinitrophenylazo)-3,6-naphthalenedisulfonic acid (B-Dnp). Many spectral changes occurred on complex formation. These resulted from short-range interactions; hence the spectra contain a great deal of information about the complex. Although complete interpretation was difficult, several main features could be delineated using the group frequency approach. Binding by antibody of the azo haptens caused them to twist about at least one of the C–N bonds of the azo group. The hapten A-Dnp was found to exist in solution in a form where the naphthyl and phenyl rings are not coplanar. On binding, a fraction twisted about the azo group in the direction of increasing planarity. In contrast, when B-Dnp is unbound, its aromatic rings are essentially coplanar, but twist out of this conformation on binding. Bound B-Dnp exists in at least two conformational states differing in extent of twist about the azo group. Differential twisting among bound haptens provides additional insight into the spread of binding free energies attributed to heterogeneity. The nitro groups in the 2 and 4 positions could be identified individually in all haptens and both underwent the same sort of shift in the NO_2 symmetrical stretching vibration on binding. This shift is consistent with a decrease in double bond character of the N–O bonds. The change in double bond character is attributed to the presence in the antibody site of a particular type of charge distribution which tends to stabilize certain resonance structures of the Dnp residue. Charge-transfer complexes were shown not to be a likely factor in causing the shifts, nor to contribute much to the binding energy.

One of the central problems of immunochemistry is to establish the identity of the amino acids comprising the active site and to elucidate their three-dimensional structural arrangement. Considerable progress has been made recently in determining site composition by techniques such as affinity labeling (Metzger *et al.*, 1963; Strausbauch *et al.*, 1971) and the correlation of variable regions in primary structure with differences in combining specificity (Ray and Cebra, 1972). However, relatively little is known about the three-dimensional structure of the combining site.

The method of choice for determining site structure of antibodies in the crystalline state would seem to be X-ray diffraction (Poljack *et al.*, 1972). For antibodies in solution one approach is to use spectroscopic methods where a spectral parameter of either the hapten or antibody changes when these moieties combine. This change would depend on the structure of the site; hence it might be used to provide some of the desired information. A number of spectroscopic methods of this sort have already been employed: circular dichroism (Rockey *et al.*, 1972), spin probes (Hsia and Little, 1971), absorption spectroscopy (Little and Eisen, 1967), and fluorescence spectroscopy (Velick *et al.*, 1960).

A new technique which may provide a sensitive tool for probing site structure of antibodies in solution as well as in the crystalline state is resonance Raman spectroscopy. Resonance Raman scattering involves an interaction of vibrational and electronic transitions with the consequence that certain vibrational modes are greatly enhanced in intensity. An essential aspect of the technique is that the wavelength of the exciting light must lie in one of the electronic absorption bands of the material of interest. When applied to colored ligands which are bound to proteins, Raman spectra of the ligand are obtained without interference from that of the protein, a poor Raman scatterer (Carey *et al.*, 1972; Strekas and Spiro, 1972; Brunner *et al.*, 1972).

Recently, it was shown that marked perturbations in Raman active bands of colored ligands occur on combination with a protein (Carey *et al.*, 1972). Since these changes result from short-range interactions between ligand and combining site, they may be useful in identifying the protein groupings vicinal to particular regions of the ligand. In addition, they may prove useful in providing insight into other aspects of the interaction. The present study applies resonance Raman spectroscopy to the interaction of dinitrophenyl haptens with specifically purified homologous rabbit antibodies in solution.

Methods and Materials

Haptens. The hapten, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalenedisulfonic acid (A-Dnp),[1] was prepared as described previously (Froese, 1968). The isomer (B-Dnp), 1-hydroxy-2-(2,4-dinitrophenylazo)-3,6-naphthalenedisulfonic acid, was purchased as the disodium salt from Eastman Organic Chemicals, Rochester, N.Y. ε-Dnp-L-lysine monohydrochloride was purchased from Schwarz/Mann, Orangeburg, N. Y. The compound 1-naphthol-2,5-disulfonic acid was a gift from Bayer Farbenfabriken A.G., Leverkusen, Germany.

† From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada, KIA OR6 (P. R. C. and H. S.), and the Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada, R3E OW3 (A. F.). *Received February 9, 1973.* Work done at the University of Winnipeg was supported by a grant from the Medical Research Council of Canada. National Research Council of Canada Publication No. 13167.

[1] Abbreviations used are: A-Dnp, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalenedisulfonic acid; B-Dnp, 1-hydroxy-2-(2,4-dinitrophenylazo)-3,6-naphthalenedisulfonic acid; Ab, antibody.

Antibodies. Anti-Dnp antibodies were obtained from rabbits immunized with protein–hapten conjugates prepared by reacting bovine γ-globulin (Nutritional Biochemicals, Cleveland, Ohio) with the sodium salt of 2,4-dinitrobenzenesulfonate (Eisen *et al.*, 1953). The immunization protocol has been described previously (Kelly *et al.*, 1971). Sera from two hyperimmunized rabbits were pooled.

Antibodies were specifically purified by the method of Robbins *et al.* (1967). Complement was removed prior to purification by precipitation of antibodies to the carrier protein, bovine γ-globulin. The purified antibody was 65% precipitable with antigen, Dnp-human serum albumin. The remaining 35% is thought to be inhibited by the eluting hapten or a column degradation product which has an absorption maximum at 363 nm.

Normal rabbit IgG was prepared by precipitation with sodium sulfate (Marrack *et al.*, 1951), followed by chromatography on DEAE-cellulose (Utsumi and Karush, 1964).

The average intrinsic association constant for the reaction of A-Dnp with specifically purified antibody, determined spectrophotometrically (Froese, 1968), was 1.75×10^5 M^{-1} in phosphate buffer, pH 6.0, $\mu = 0.10$, at 25°. The antibody preparation was heterogeneous as indicated by the nonlinearity of the binding curve.

For A-Dnp and B-Dnp antibody–hapten solutions for Raman studies were made up by adding 1 vol of a 10^{-4} M hapten solution to 9 vol of antibody solution with an available site concentration of 6.85×10^{-5} M. Both solutions were in the phosphate buffer, pH 6.0, $\mu = 0.10$, used in binding experiments and for all Raman studies. Final concentrations in experiments with Dnp-lysine were 5×10^{-5} M for hapten and 6.17×10^{-5} M for antibody sites. At the final concentrations employed with A-Dnp, 10^{-5} M for hapten and 6.17×10^{-5} M for antibody sites, more than 90% of the hapten was bound. A similar value was assumed with B-Dnp and Dnp-lysine, previous experience having shown their affinity for anti-Dnp antibodies to be greater than that for A-Dnp at pH 6.0. The utility of this assumption was subsequently justified by the absence of bands in the spectra of all antibody–hapten solutions attributable to unbound hapten.

Because conditions were employed in experiments with A-Dnp and B-Dnp where antibody concentration exceeded that of hapten by a factor of 6.17 only approximately 15% of the antibodies will have combined with hapten. Because of the presence of heterogeneity, these are probably the sites having the highest affinity for the haptens. In experiments with Dnp-lysine, a larger population of antibody sites are bound, the hapten:antibody ratio being 0.81.

Normal rabbit γ-globulins under the conditions employed for antibodies produce only insignificant shifts in absorption spectra of the haptens employed (Froese, 1968); hence interference from nonspecific binding was not expected. This expectation was confirmed in a control experiment with B-Dnp where normal rabbit γ-globulins were substituted for antibodies, and Raman spectra were obtained which were identical with those in buffer alone.

Raman and Infrared Spectra. Raman spectra for A-Dnp were obtained using a Spex 1400 spectrometer with 100–1000 mW of Ar^+ laser power at 4880 Å. The lower power levels were employed to obviate heating effects in the protein solutions. Raman spectra for the other two haptens were obtained using a Jarrell-Ash 25–400 system with 50–100 mW of the 4579 Å line. One spectrum of Dnp-lysine employed the 6471-Å Kr^+ line.

The sample cell for antibody–hapten solutions was a quartz capillary of 5 μl volume. The laser beam entered along the capillary axis and was accurately positioned and focused inside by mounting the capillary in a goniometer head. The absence of photochemical decomposition of the haptens or photoisomerization about the azo group of A-Dnp and B-Dnp was indicated by the invariance with time of irradiation of the intensities of all spectral features, including a group frequency for the aromatic azo linkage. Thermal effects of the laser beam were minimal since only a small thermal lens effect (Leite *et al.*, 1967) was observed in aqueous hapten solutions. A rotating Raman cell was used for solid hapten samples and for charge transfer studies. For solids the hapten was mixed with 99% by weight of KBr and pressed in the cell (Kiefer and Bernstein, 1971).

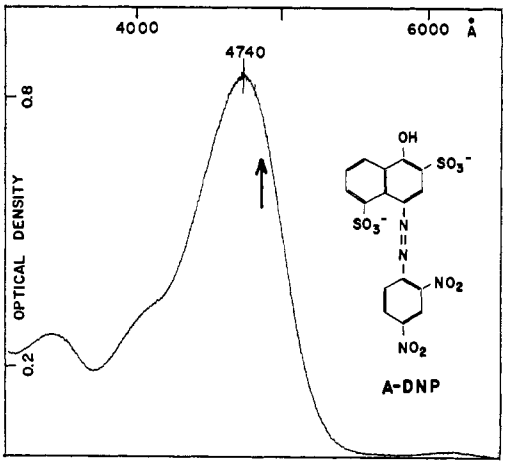

FIGURE 1: The absorption spectrum of ~3.3×10^{-5} M 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalenedisulfonic acid (A-Dnp) at pH 6.0. The laser Raman excitation wavelength (4880 Å) is indicated by an arrow.

All spectra were repeated at least three times to ensure reproducibility. For hapten–antibody spectra three or more separate mixtures were investigated resulting in at least ten spectra of each bound hapten.

Infrared spectra were obtained using a Perkin-Elmer Model 257 spectrometer. Absorption spectra were measured using a Cary 14 spectrophotometer.

Results

Choice of pH. Haptens such as A-Dnp and B-Dnp can be bound with their phenolic residue either protonated or unprotonated (Froese, 1968; Froese *et al.*, 1969; Metzger *et al.*, 1963). Since different Raman spectra would be given by these two forms, complications in interpretation would arise if they were obtained simultaneously. To avoid such complications in this initial study conditions were chosen where spectra of only protonated forms were obtained selectively. This was accomplished through appropriate choice of pH (6.0) and excitation wavelength. At pH 6.0, 95% of free and bound A-Dnp is protonated, the pK of the phenolic residue on the unbound material being 7.69 (Froese, 1968). Binding of B-Dnp causes a shift in phenolic pK from 6.52 to approximately 9 (Metzger *et al.*, 1963). Thus, at pH 6.0 the phenolic group of bound B-Dnp is protonated. The more abundant species of unbound B-Dnp at this pH is the protonated form (Figure 2, Metzger *et al.*, 1963), and since the excitation wavelength (4579 Å) is far removed from the absorption maximum of the unprotonated form (~6000 Å) the Raman spectrum of

TABLE I: Raman and Infrared Line Positions for A-Dnp.[a]

Raman (cm^{-1})				Infrared (cm^{-1})
Hapten–Ab	Hapten–H_2O	Hapten–D_2O	Solid Hapten–KBr	Solid Hapten–KBr
1644 (b)	1649 (b) (0.35)	1649	1649	1648
1611	1613 (0.35)	1618	1617	
		1608		1603
1545 (sh)	1546 (sh)	1564	1559	1592, 1580 (sh), 1560 (sh)
1519	1521 (sh)	1520 (sh)	1518 (sh)	1521 (sh)
1508	1508 (0.35)	1512	1511	1507
		1492 (sh)		
1453	1452 (0.5)	1455	1454	1452
	1374 (sh)	1377 (sh)	1382	1378
1357	1357 (0.4)	1359	1359	
1335	1345 (sh)	1345 (sh)	1347 (sh)	1343
1310	1319 (0.35)	1321	1320	1319
1272	1279	1287	1282	1280
1264		1271		1267
1227 (b)	1230 (b)		1237 (b)	1232, 1202, 1183, 1170
1135 (b)	1134	1139		1151, 1134, 1121, 1104
1075		1071		1067 (sh)
1030 (b)	1035 (0.3)	1048	1035	1048 (sh), 1032
		1001 (b)	1023	1024 (sh)
928	928 (0.5)	928	926	928, 910, 890
872				865, 851
835	839 (0.35)	839	836	837
			794	795 (sh), 785, 749
729	736 (0.35)	725	729	732 (sh)
		691		
	563 (0.45)	562	560	
	475 (0.45)	476	476	
	453 (sh)	450		
397	398 (0.5)	395		
	377 (sh)			

[a] sh = shoulder, b = broad. Depolarization ratios are shown in parentheses.

this species is of negligible intensity compared to that of the protonated form. By the same token, using blue excitation wavelengths, the greater enhancement of the Raman spectra of the protonated over the unprotonated A- and B-Dnp species means that in D_2O solutions only spectra of the protonated forms were obtained, even allowing for differences in degree of dissociation due to substituting D_2O for H_2O.

Spectral Assignments. Absorption spectra of the haptens in pH 6.0 buffer are shown in Figures 1–3. Raman spectra of A-Dnp in H_2O, D_2O, in the solid phase, and bound to the antibody are compared in Figure 4. For free and antibody-bound A-Dnp in H_2O an expansion of a region of major interest, 1200–1700 cm^{-1}, is shown in Figure 5. Figure 6 presents an infrared spectrum of A-Dnp in a KBr pellet: line positions are tabulated in Table I. Raman spectra of free and bound B-Dnp are compared in Figure 7. Figure 8 shows the Raman spectra of Dnp-lysine. Line positions for B-Dnp and Dnp-lysine are tabulated in Tables II

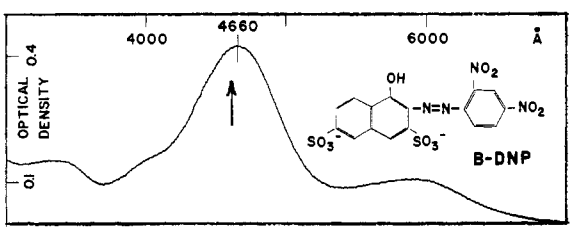

FIGURE 2: The absorption spectrum of ~1.7 × 10^{-5} M 1-hydroxy-2-(2,4-dinitrophenylazo)-3,6-naphthalenedisulfonic acid (B-Dnp) at pH 6.0. The laser Raman excitation wavelength (4579 Å) is indicated by an arrow.

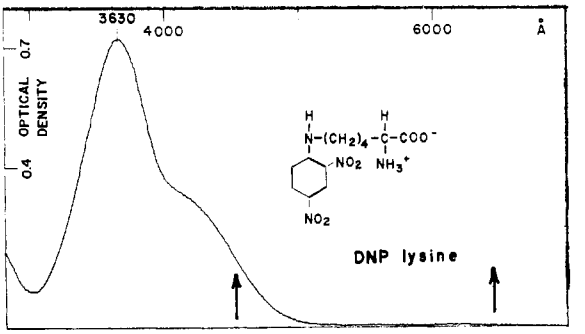

FIGURE 3: The absorption spectrum of approximately 10^{-4} M ε-2,4-dinitrophenyllysine (Dnp-lysine) at pH 6.0. The laser Raman excitation wavelengths (4579 and 6471 Å) are indicated by arrows.

TABLE II: Raman and Infrared Line Positions for B-DNP.

Raman (cm^{-1})			Infrared (cm^{-1})
Hapten–Ab	Hapten–H_2O	Hapten–D_2O	Hapten–KBr
1650 (sh)	1641 (0.4)		1642, 1648 (sh), 1654 (sh)
1618	1607 (0.4)		1597, 1622
1516	1518 (0.35)		1495, 1512, 1525 (sh)
1435, 1420 (b, sh)	1417 (0.3)	1420	1411
1350[a]	1353[a] (sh) (0.3)	1360	1350
1312, 1329 (sh)	1320 (sh)	1340 (s)	1325
1274	1277 (0.35)		1260, 1284
1228			1242
1210	1209	1220 (b, sh)	1208
1140	1147 (0.3)	1145	1111, 1140
1076	1072 (0.25)	1080	1045, 1062, 1071
1003		1011	1028
926	929 (0.5)	923	910
878	877		
835	840 (0.5)	838	818, 839, 848
	740 (sh)		747, 772
	700	710 (b)	652, 671, 691
	612 (0.5)	608	
510, 530	530 (b) (0.3)	532	
398, 454	389 (0.4)	392, 452	
	335	337	

[a] Feature around 1350 complicated by strong laser line at 1344.8 cm^{-1}. Information in D_2O is limited by high background. Depolarization ratios are quoted in parentheses; sh = shoulder, b = broad.

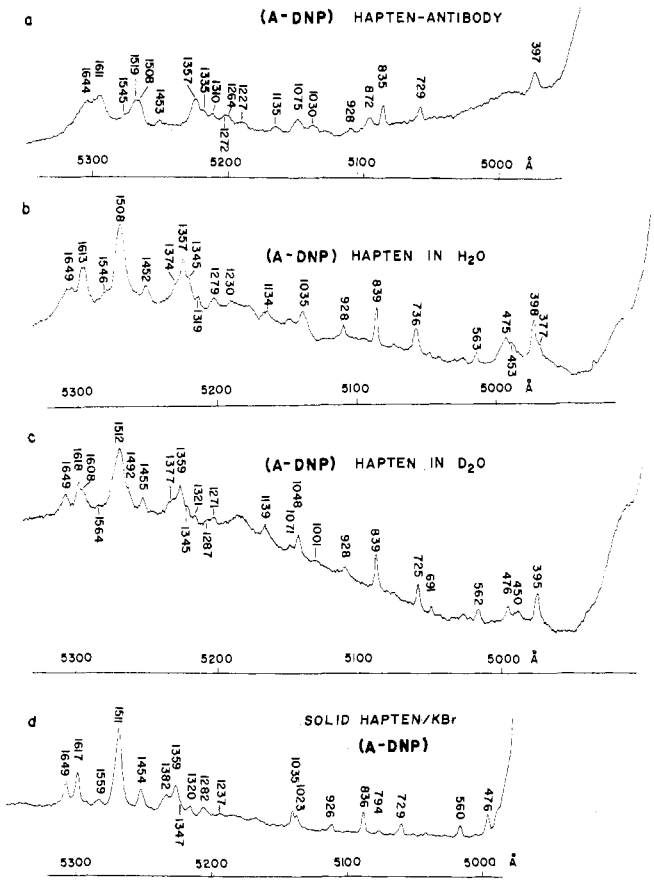

FIGURE 4: Laser Raman spectra of 10^{-5} M A-Dnp at pH 6.0: (a) bound to antibody, (b) in H_2O, (c) in D_2O, and (d) 1% by weight in KBr; spectral slit width, 6 cm^{-1}; 4880-Å excitation.

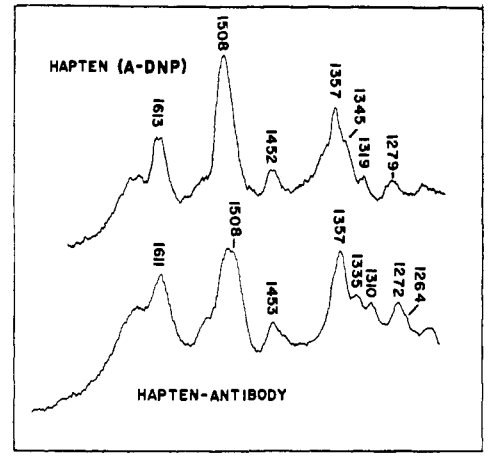

FIGURE 5: Comparison of A-Dnp and A-Dnp–antibody spectra between 1250 and 1750 cm^{-1}; 4880 Å excitation; spectral slit width, 6 cm^{-1}.

and III, respectively. Line positions and intensities for 1-naphthol-2,5-disulfonic acid are given in Table IV.

Complicated changes in Raman spectra occur on binding. Using B-Dnp as an example (Figure 7) and selecting some of the more pronounced changes, bands change in intensity (*e.g.*, 1350 cm^{-1}, *cf.* 1147, 926 cm^{-1}) and shift (1417 → 1435 cm^{-1}), and new features appear (1003 cm^{-1}). These manifold changes indicate the spectra contain a great deal of information concerning the antibody–hapten complex. At present, complete interpretation of Raman spectra of the haptens alone is a formidable task. As a result, complete interpretation of the changes on binding can also be expected to be difficult.

TABLE III: Raman and Infrared Line Positions for Dnp-Lysine.[a]

Raman (cm^{-1})			Infrared (cm^{-1})
Hapten–Ab (4579 Å)	Hapten–H_2O (4579 Å)	Solid Hapten (6471 Å)	Dnp-Lysine–KBr
	1593 (sh)	1619 (0.3)	1590, 1619, 1739
	1533 (b)	1528 (b)	1505, 1528
	1443 (0.25)	1432	1427, 1457
1372	1374 (0.4)	1363 (sh)	1363
1331	1340	1333 (0.3)[b]	1335
	1314 (0.4–0.5)	1316 (0.3)[b]	1320
	1281 (0.4–0.5)	1268 (sh) (0.5)	1290 (sh)
	1248 (sh) (0.4–0.5)	1243 (sh) (0.6)	1199, 1240
1140	1151	1138	1139
	1080 (<0.1)		1056, 1081, 1091
1033	1034		
	993		
	929		925
875	877 (0.25)		
828	835	832 (0.1)[b]	820, 832
720	714 (0.3), 794 (b)	705	703, 748
	523 (b)	391 (b)[b]	
	394 (0.3)	98 (sh)	

[a] In the spectrum of Dnp-lysine + Ab information was limited by high spectral background. The slope of the background makes all measured (quoted) peaks too high in cm^{-1}. [b] These lines come from the Dnp moiety and are common to all three haptens; 832 and 391 may be of utility in future work. Depolarization ratios for the most intense bands appear in parentheses; those quoted for 6471-Å excitation were obtained from a nearly saturated aqueous solution. The 6471-Å line was used to obtain Raman spectra under conditions far from resonance. Mixtures of Dnp-lysine–KBr gave intense fluorescence and were not used.

TABLE IV: Raman Spectrum of 1-Naphthol-2,5-disulfonic Acid.[a]

805 (1)	1358 (10)
1043 (4)	1460 (1.5)
1070 (sh) (0.5)	1577 (2)
1242 (0.5)	

[a] Exciting line 4880 Å. Nearest electronic absorption maximum is at 3015 Å; thus, the spectrum was obtained under nonresonance conditions. Relative intensities are shown in parentheses; sh = shoulder.

Nevertheless, a good deal of information can be obtained using group frequencies, the main approach used in this study. In essence, certain spectral features are first identified with molecular groupings in the hapten. Then, by examining the hapten under a variety of conditions and using the wealth of group frequency data obtained from infrared spectroscopy, the effect of antibody on these groupings is delineated.

AROMATIC AZO BANDS. Cis or trans aromatic azo linkages normally have a readily assignable band in both Raman and infrared spectra (Hacker, 1965; Kübler *et al.*, 1960). In the trans form, when the aromatic rings are coplanar, the N=N stretching vibration is in the region 1380–1440 cm^{-1}. However, when the rings are twisted out of the coplanar conformation the N=N stretch frequency increases. This change results from a decrease in conjugation throughout the π-electron system with a concomitant increase in bond order in the N=N linkage. In the cis conformation the expected frequencies are in the 1500-cm^{-1} region.

Spectra for unbound A-Dnp indicate that the aromatic rings are severely twisted out of a planar conformation. There was no prominent band in the trans azo region, and that at 1452 cm^{-1} was assigned to the N=N stretching vibration of a twisted form. This assignment is supported by space-filling molecular models (Corey–Pauling–Koltun (CPK)) which show that there would be strong steric interactions between the 5-sulfonate residue and the Dnp ring. However, these can be reduced by twisting of the aromatic rings about at least one of the C—N bonds of the azo groups. The models suggest the dihedral angle is closer to 90° than to 0°. The cis azo conformation does not seem possible in this compound because of extensive steric hindrance.

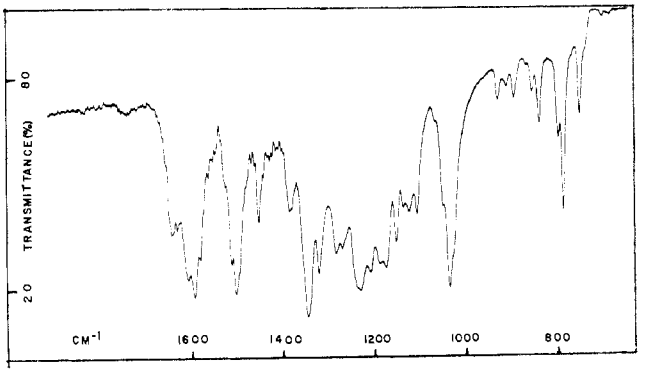

FIGURE 6: Infrared spectrum of A-Dnp in a KBr pellet.

TABLE V: Infrared and Raman NO_2 Symmetric Stretch Frequencies.[a]

	Infrared Unbound in KBr		Raman Unbound in H_2O		Raman Bound to Antibody	
Hapten	4-NO_2	2-NO_2	4-NO_2	2-NO_2	4-NO_2	2-NO_2
Dnp-lysine	1320	1335	1314	1340	<1314 (na)	1331
A-Dnp	1319	1343	1319	1345 (sh)	1310	1335
B-Dnp	1325	1350	1320 (sh)	1340–1350 (na)	1312	1329

[a] cm^{-1}; sh = shoulder; na = position not accurate.

Confirmation of the above assignment for the azo band of A-Dnp is provided by the B-Dnp spectra. As indicated by space-filling models, steric hindrance is much less important in this compound and the aromatic nuclei can assume a more planar conformation. In both Raman and infrared spectra the 1453-cm^{-1} band found in A-Dnp is absent and is replaced by one at 1417 cm^{-1} in the "expected" range for unhindered trans azo groups.

PHENOLIC GROUP AND NAPHTHYL RESIDUE. A group frequency for the phenolic OH group could not be utilized in the Raman spectra. Hydrogenic modes are not intensity enhanced under resonance Raman conditions and hence are of negligible intensity compared to those which are enhanced. In addition, although features in the infrared spectra of phenols have recently been assigned as approximating the C–OH stretch (Bist *et al.*, 1967; Pinchas, 1972) their utility as group frequencies is not established. However, spectral regions sensitive to changes at the phenolic residue could be identified by taking the spectra in D_2O. At pH 6.0, the only change produced in this way is deuteration of the phenolic residue and thus spectral changes result either from naphthalenic ring modes being vibrationally coupled to the –C–OH group or from observation of a band having a high degree of "C–OH character." Principally these spectral regions are near 730, 1040, and 1280 cm^{-1} for A-Dnp. It is noteworthy that a feature near 1260 cm^{-1} in a series of phenols has been identified as "approximately C–OH" (Bist *et al.*, 1967; Green *et al.*, 1971; Pinchas 1972), in agreement with our observation of the deuterium-sensitive 1280-cm^{-1} feature. For B-Dnp, the D_2O sensitive regions are near 925, 1005, and 1075 cm^{-1}. Small changes in the B-Dnp spectrum at higher wave numbers are obscured by a high background.

The Raman spectrum of 1-naphthol-2,5-disulfonic acid (Table IV) shows a strong peak at 1358 cm^{-1}. Although this partially masks the NO_2 stretching bands (next section) it is important to note that this feature comes from a part of the hapten vibrationally distinct from the NO_2 groups.

AROMATIC NITRO GROUPS. The NO_2 symmetric stretching vibrations of each of the nitro groups could be identified individually in all three haptens (Table V). Spectral analysis in this instance was greatly aided by the fact that the Dnp residue is the only chromophore in Dnp-lysine; hence all features in a resonance Raman spectrum must be closely linked vibrationally with this residue. From the infrared spectra of Dnp-lysine the 4- and 2-nitro group symmetric frequencies were assigned to the 1320- and 1335-cm^{-1} peaks, respectively (Bellamy 1968; Conduit, 1959). As a result the 1316- and 1333-cm^{-1} features in the 6471-Å spectrum of solid Dnp-lysine and the 1314- and 1340-cm^{-1} features in the 4579-Å spectrum of aqueous Dnp-lysine could unambiguously be identified as being due to the 4- and the 2-nitro groups, respectively. Slight differences between Raman and infrared frequencies are due to experimental error and environmental factors, Raman spectra being obtained with aqueous and solid Dnp-lysine, infrared spectra being obtained from a KBr pellet.

For A-Dnp the relevant NO_2 frequencies were assigned to the 2- and 4-nitro groups as 1345 and 1319 cm^{-1} on the basis of similar features in infrared spectra at 1343 and 1319 cm^{-1}.

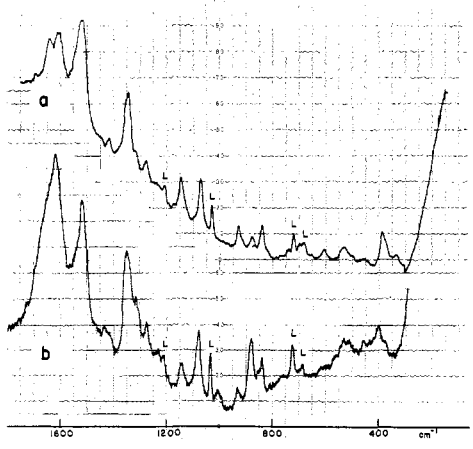

FIGURE 7: Raman spectra of 10^{-5} M B-Dnp at pH 6.0: (a) in H_2O (b) bound to antibody; 4579-Å excitation; spectral slit width, 8 cm^{-1}. Water and phosphate make contributions at approximately 1650 and 880, 990, and 1080 cm^{-1}, respectively; L = non-Raman lines emanating from the laser plasma.

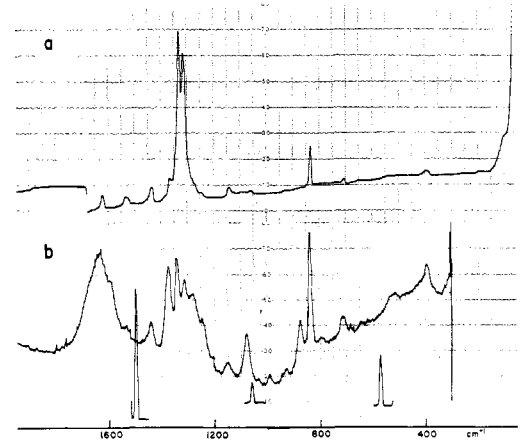

FIGURE 8: Raman spectra of Dnp-lysine: (a) solid using 6471-Å excitation, (b) 10^{-4} M in H_2O using 4579 Å; spectral slit width, 8 cm^{-1}. Calibration peaks can be seen near the cm^{-1} scale.

and the Dnp-lysine assignments. In the Raman the nitro peak at 1345 cm^{-1} is partially marked by a band at 1357 cm^{-1} (most probably emanating from the naphthyl residue (previous section)) which is not present in the infrared spectrum. In B-Dnp the feature in the infrared at 1325 cm^{-1} is readily correlated with the line in the Raman at 1320 cm^{-1}. However, the line in the infrared at 1350 cm^{-1} due to the 2-NO_2 group is masked in the Raman by a strong laser line at 1344.8 cm^{-1} and the presence of the naphthyl mode which is absent in the infrared.

The antisymmetric NO_2 stretching frequencies could not be employed since they did not appear to be resonance Raman active. The antisymmetric nitro stretching frequency has been well characterized in infrared spectra (Bellamy, 1958a, 1968) and in aromatic compounds normally occurs between 1490 and 1530 cm^{-1}. However, Raman spectra of Dnp-lysine did not contain any intense spectral feaures in this region. Raman spectra of A- and B-Dnp do contain intense features at 1510 and 1516 cm^{-1} which could conceivably be assigned to the antisymmetric vibrations. However, such an assignment is invalid because of the Dnp-lysine spectra and, also, on intensity and depolarization ratio intensity grounds (Behringer, 1967b). Intensities of the NO_2 symmetric stretch frequencies are greater than those for the antisymmetric stretch under nonresonance conditions and the disparity will be more pronounced in a resonance Raman spectrum (Behringer, 1967a, and references therein). Consequently the greater intensity of the 1510 features compared with those at 1345 and 1319 cm^{-1} makes it very improbable that the former are due to the antisymmetric NO_2 stretch. However, it is to be noted that changes occur in this region on binding, and that eventual assignment of frequencies around 1500 cm^{-1} may be useful in describing the properties of the complex.

C–N Vibrations. The C–N vibrations of aromatic nitro groups are not well characterized (Bellamy, 1958b). The C–N vibrations of the azo bonds have been assigned in sterically uncrowded azobenzene and azonaphthalene derivatives to the 1140–1150-cm^{-1} region (Hacker, 1965). In the only molecule of this type studied, B-Dnp, the prominent 1147-cm^{-1} band is assigned to the azo C–N stretching mode.

Interpretation of Spectral Changes on Binding Hapten to Antibody. Aromatic azo groups. Changes in the N=N symmetric stretch frequencies indicate that the azo haptens change conformation on binding. The position of the maximum for A-Dnp does not change (1453 → 1452 cm^{-1}, Figure 5), but the peak broadens to the low wave-number side. With B-Dnp the 1417-cm^{-1} band nearly disappears (Figure 7), to be replaced by one at 1435 cm^{-1} with a shoulder at 1420 cm^{-1}. The change in A-Dnp is attributed to slight twisting about at least one of the C–N bonds of the azo group of a fraction of the bound haptens. The change with B-Dnp is attributed to binding in at least two conformations differing in their extent of twisting about the azo group C–N bonds. The direction of twist in A-Dnp is in the direction of increasing planarity, for B-Dnp torsion about the C–N bond forces the aromatic nuclei out of planarity. Taking the magnitude of the shifts to be proportional to extent of twisting, binding twists B-Dnp more than A-Dnp. Twisting of the bound haptens to varying extents is attributed to the presence of antibody sites differing in structure, that is, antibody heterogeneity.

Twisting about the azo C–N bonds of B-Dnp can be directly monitored using the C–N stretching vibration at 1147 cm^{-1}. Twisting about this linkage increases single bond order with a resultant decrease in frequency. Conjugation through the C–N bond is reduced by twisting and a reduction of resonance Raman intensity is expected. In agreement with these predictions, upon complex formation the 1147-cm^{-1} band moves to 1140 cm^{-1} with a significant decrease in intensity compared to, for example, the 1350- and 1520-cm^{-1} features (Figure 7). Since the spread of frequency is less than that for the N=N linkage, "twisted" and "planar," the effects of heterogeneity are not so readily visible. Such effects might be studied by a band-shape analysis.

In the case of Dnp-lysine the poor quality of the antibody–hapten Raman spectra prevent conclusions being drawn about heterogeneity in this system.

Phenolic and naphthyl residues. Twisting about the azo bonds suggests interactions occur with the naphthyl residue as well as with the Dnp group. Evidence for naphthyl residue interactions is provided by changes in regions sensitive to D_2O with both A- and B-Dnp. Three types of interactions could lead to these spectral changes: direct contact with (1) the phenolic residue, (2) naphthalene ring atoms, and (3) the sulfonate ions. These may occur singly or together, and the present data cannot determine which of the various possibilities occurs. Interaction with the sulfonate ions must be considered explicitly since their interaction with a positive charge would be expected to produce changes in naphthalenic ring modes, by analogy with Methyl Orange–bovine serum albumin (Carey *et al.*, 1972).

Aromatic nitro groups. The two nitro groups of all haptens used suffer similar changes on binding, the symmetric stretch frequency decreasing by about 10 wave numbers (Table V). Thus, both groups undergo similar perturbations. The spectral changes are clearest in A-Dnp; the 1345 band (2-NO_2 group) shifts to 1335 cm^{-1} and the 1319 band (4-NO_2 group) shifts to 1310 cm^{-1}. In B-Dnp the shift of the 1320 band to 1312 cm^{-1} is unequivocal. The 1340–1350-cm^{-1} band (4-NO_2 group), masked in the free hapten, probably reappears as the 1329-cm^{-1} shoulder on binding. For B-Dnp the 2-NO_2 may form a strong intramolecular H bond to the phenolic residue (Urbanski and Dabrowska, 1959); however, from the infrared data the similarity of the NO_2 symmetric stretches of A- and B-Dnp suggests this is not the case. X-Ray data on the crystal structure of B-Dnp could be definitive on this point.

Interpretation of the Dnp-lysine spectra was rendered difficult by the fluorescence the hapten exhibited after binding to the antibody site. The fluorescence lay in the region above 5000 Å and was of sufficient intensity to mask some of the Raman lines. Such fluorescence has been attributed to freezing out of rotational modes on binding (Winkler, 1962; Oster and Nishijima, 1956). Absorbed quanta cannot then be dissipated by transfer to these modes and are re-emitted as fluorescence. However, in spite of the background fluorescence, the NO_2 shifts could be monitored and essentially similar effects were observed as with the azo haptens. The stronger NO_2 band, 1340 cm^{-1}, could be followed and this shifted to 1331 cm^{-1}. In some spectra the weaker band (1316 cm^{-1}) could be seen to lower wave numbers, but it could not be positioned with any accuracy. Shifts in Dnp-lysine are considered accurate to ± 5 cm^{-1} and to ± 2 cm^{-1} in the other haptens.

Although the value of the symmetric NO_2 stretch as a group frequency is reduced by coupling effects, probably involving C–N and ring modes, it is sensitive to perturbations. Changes due to steric effects (Van Veen *et al.*, 1957) or isotopic substitution (Pinchas *et al.*, 1964) are detectable and are consistent with concepts of changing bond order or effective mass.

Thus, the observed shifts of the NO_2 frequencies to lower wave numbers are attributed to a decrease in bond order or double bond nature in the N–O bonds. If the valence structure of the Dnp residue is considered to have contributions from the forms shown in Figure 9, then II to IV make a larger contribution to the bound hapten. It is suggested that these particular structures are stabilized in the bound form by the presence of a particular type of charge configuration in the active site. The possibilities for this charge configuration are (i) positive charges close to the oxygens of the nitro groups, (ii) permanent dipoles oriented with their positive charge close to the nitro group oxygens, and (iii) at least one integral or partial negative charge vicinal to positions 1, 3, or 5.

Several possible alternative causes of the spectral shifts can be ruled out. Gross twisting alone, >30°, about the C–N bonds of the nitro groups out of the plane of the benzene ring would cause an increase in symmetric stretching frequency, rather than the decrease observed. It would also cause a considerable reduction in the intensity of the nitro peaks (Shorygin and Il'icheva, 1958). Bending of the NO_2 group out of the plane of the benzene ring would increase the double bond character of the N–O bonds and increase the NO_2 symmetric stretch frequency. Although hydrogen bonding to NO_2 or dielectric effects may be present, such effects cannot by themselves account for the shift since they usually produce only small, often immeasurable, changes (Bellamy, 1968). Inductive effects through aromatic electrons in A- and B-Dnp where a perturbation in the naphthyl residue is transmitted to the Dnp residue do not seem likely for two reasons. (1) Interactions at the phenolic residue do not affect the NO_2 frequencies as shown by D_2O experiments and (2) comparable shifts occur in Dnp-lysine where there is no aromatic nucleus coupled to the Dnp residue.

However, it is difficult to determine if there are several superimposed effects with competing effects on the NO_2 shifts. For instance, it is difficult to determine if the observed downward shift of 10 wave numbers is due to a charge configuration in the antibody site, which by itself might cause a downward shift of 15 cm^{-1}, superimposed on a small twist of the NO_2 group which, by itself, could cause an upward shift of 5 wave numbers. Despite this difficulty, it is clear that the preponderant effect could be due to charge distributions of the sort proposed.

Charge-Transfer Complexes. A charge-transfer complex between dinitrophenyl haptens and tryptophan in the binding site has been proposed to play a role in the hapten–antibody interaction (Little and Eisen, 1967). The present study suggests that such complexing probably does not provide a large portion of the free energy of binding, nor cause the NO_2 shifts. Indole (0.05 M) and *s*-trinitrobenzene (0.05 M) were found to interact in chloroform to produce a charge-transfer band with a broad maximum in the absorption spectrum between 400 and 500 nm. Resonance Raman spectra of the complex showed changes only in the intensity of the NO_2 symmetric stretching band and no evidence for a shift in band position. Thus, in all likelihood, the NO_2 shifts upon complexing to antibody are probably not caused by a charge-transfer interaction. Band shifts could not be detected in other parts of the charge-transfer complex spectrum although several features were masked by solvent peaks. These result are consistent with the finding that band shifts cannot be detected in solution spectra of the trinitrobenzene–anthracene charge-transfer complex (Larkindale and Simpkin, 1972).

The reasons that charge-transfer complexes are considered not to make a large contribution to the free energy of binding are the following. Any charge-transfer complex between an indole residue of a tryptophan in an antibody site and the dinitrophenyl group would probably be weaker than that between indole and trinitrobenzene since only very much smaller spectral charges are produced in the absorption spectrum of bound Dnp-amino acids (Little and Eisen, 1967) or Dnp-protonated phenol haptens (Froese, 1968). In addition, since formation of a charge-transfer complex between indole and trinitrobenzene does not detectably perturb the ground vibration states of the constituents, the free energy of binding is probably small compared to antibody–hapten binding where the ground vibrational states are perturbed, as indicated by the many changes in vibrational spectra observed in the present study.

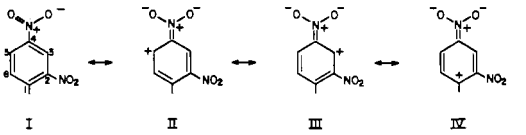

FIGURE 9: Resonance structures for the dinitrophenyl ring. Structures shown only for the 4-nitro group. Similar structures can also be written for the 2-nitro group.

Discussion

The salient results of the present study may be summarized as follows. (1) The azo haptens may twist about the C–N bonds of the azo linkage when complexed with antibody. (2) For a given hapten, the degree of twisting is not the same in all complexes. (3) The two nitro groups of the Dnp determinant suffer identical perturbations and, moreover, these perturbations are the same irrespective of the hapten in which the Dnp group is incorporated. Some implications of these results are discussed in detail below.

Twisting of Hapten. Differential torsion about chemical bonds in the bound form of the hapten is attributed to antibody heterogeneity. It is considered that the antibodies employed contained sites differing somewhat in structure. Hapten twisting occurred because twisted forms fitted best into some sites.

Torsion about chemical bonds requires energy, and when torsion occurs in the course of a protein–ligand interaction, the associated energy must necessarily be reflected in the standard free energy of reaction. The net effect may range from making the standard free energy less favorable to making it more favorable. If torsion occurs and the resultant antibody–hapten contacts yield an interaction energy identical with that for untwisted haptens, the free energy of reaction would be less favorable. The term interaction energy is used here to represent that due to the van der Waals, electrostatic, and hydrogen bond interactions involved in hapten–antibody complex formation. However, torsion could also occur under circumstances where the interaction energy would be greater or lesser than that for sites binding untwisted haptens. This difference in energy could be larger than that required for torsion. Greater interaction energies could then make the standard free energy of reaction more favorable and *vice versa*.

On the basis of the above discussion the demonstration that hapten twisting can occur provides additional insight into the energetics of antibody–hapten interactions. The results suggest that the range of free energies or binding constants

which produce curvature of binding isotherms reflects, at least in part, twisting of the hapten during the binding process.

It can be shown readily that torsion about azo bonds is associated with energies of the correct magnitude to be considered a factor contributing to binding isotherm curvature. Azo compounds usually exist in two states, cis and trans. In azobenzene the barrier to rotation, taken equal to the activation energy for cis–trans isomerization, is ~23 kcal (Hartley, 1938). The difference in enthalpy between cis and trans forms is ~10 kcal (Corruccini and Gilbert, 1939). The torsional energy, U_{tor}, may be expressed as (Scott and Scheraga, 1965)

$$U_{tor} = \frac{U_b}{2}(1 - \cos n\chi) + \frac{U_e}{2}(1 - \cos n\chi)$$

where U_b is the barrier height, U_e is the energy difference between minima, and n depends on the symmetry of the transition. The angle of rotation is given by χ. Hence

$$U_{tor} = \frac{23}{2}(1 - \cos 2\chi) + \frac{10}{2}(1 - \cos 2\chi)$$

For displacements of 5 and 10°, the torsional energies are, respectively, 0.25 and 1.0 kcal. The overall mean free energy for the interaction is 7.2 kcal. Assuming this value closely approximates that for combination without hapten deformation, it is obvious that the overall free energy is sufficiently large to allow appreciable interaction even if some twisting occurs. Furthermore, small rotations (up to ~10°) require energies comparable to those causing the spread of free energies characteristic of heterogeneity. For example, for a Sips "a" value of 0.7, 75% of the sites lie within the range 0.16–6 K_0, which corresponds to a spread in free energy of 1.0 kcal at 25°.

It is difficult at present to relate quantitatively calculations of the above type to hapten–antibody interactions. One reason is that the changes in interaction energy due to hapten twisting are not known. A second reason is that the exact values of the torsional angle or range of angles are not known. In principle these angles are obtainable from Raman data. However, additional information is required which is not yet available. This information is, in essence, a calibration curve relating the N=N symmetrical stretching frequency with the dihedral angle of associated aromatic residue. Determination of this curve requires Raman studies on a series of solid azo compounds with known crystal structure. Although data of this sort are available for biphenyl derivatives, there is none yet for the aromatic azo linkage.

There is an additional factor making difficult the quantitative correlation of hapten twisting with standard free energy of reaction. However, in principle, this factor comes up only because of the particular conditions used in present experiments. The spectroscopic studies were carried out in the antibody excess region (~15% of sites bound). Determination of binding data in this region required measurement of hapten concentration by spectrophotometric means in the range below 10^{-6} M. Such measurements are fraught with difficulties yielding low accuracy and poor precision. Thus, curvature of the binding isotherm in the concentration region where the Raman studies were carried out could not be properly evaluated.

It is noteworthy that since it has been shown that haptens can twist on combining with the antibody site, consideration must also be given to the possibility that the site can also change structure. This view is prompted by the fact that energy barriers for torsion about many bonds in proteins are relatively small (<4 kcal mol^{-1}) (Scheraga, 1968). Such protein changes would, of course, also contribute to the standard free energy. Recent evidence for the occurrence of changes in antibody structure on combination with antigens has been presented by Liberti *et al.* (1972a,b).

The experimental manifestation of heterogeneity in antibody–hapten interactions has been attributed to the presence of sites (1) which bind haptens in different orientations, (2) which bind to different regions of haptens, (3), which are directed partly against the linkage between hapten and carrier protein (Pressman and Grossberg, 1968). The latter view is considered to represent the main factor responsible for the differences in site structure in the preparation employed. The immunizing antigen employed consisted of bovine γ-globulins to which approximately 50 Dnp residues per molecule had been coupled. Therefore, it is likely the methylene chains of the various lysyl residues differed in conformation due to differences in microenvironment, and also that different adjacent amino acid residues contributed to the various immunogenic determinants. The naphthyl residue of the azohaptens would be expected to interact with the linkage region of the immunogenic determinants.

A point of interest is that it has been shown that heterogeneity can occur even on immunization with a protein conjugate bearing only a single haptenic residue (Eisen *et al.*, 1964). A hypothesis to account for this fact is provided by the evidence that antibody-bound haptens can be twisted out of their solution conformation. Since twisting may require only a small amount of energy relative to the standard mean free energy of binding, then complexes of immunizing antigen and immunocompetent cells may involve deformed haptens. Twisting would be a consequence of slight differences in structure of receptor sites on immunocompetent cells. Antibodies would therefore be elicited by sites which preferentially bind deformed haptens and also by other sites which preferentially bind undeformed haptens with the result that the total antibody population would appear heterogeneous. Of course, this phenomena could occur with each haptenic residue on immunizing conjugates containing many such residues.

In principle, twisting can also occur about the C–N of the nitro group. Such twisting could even be expected, the 8 kcal involved (Yan *et al.*, 1968) being less than the ~20 kcal for azo bonds. In fact, nitro groups are easily thrown out of the plane of the aromatic ring by noncovalent interactions such as occur in crystals. Twisting of 32° has been observed recently (Seff and Trueblood, 1968). Twisting can also be produced by steric factors (Grant and Richards, 1969; Trotter, 1959). Moreover, bending out of the plane can be produced by noncovalent interactions in crystals (Abrahams, 1950).

The absence of evidence for gross twisting or bending suggests the hapten NO_2 groups may have the same orientation in the crystalline state, bound to the antibody, and free in solution. Unfortunately determination of this angle, or angles if those for the 2 and 4 positions differ, is not possible at present since appropriate crystal-structure data are not yet available. However, since NO_2 spectral properties are essentially identical in all three haptens, it seems unlikely that the NO_2 groups would all be twisted to the same extent in the solid phase for all three compounds. Thus, it is likely they all have essentially the same, coplanar conformation. If this is true, then antibodies are elicited by the planar forms

of the NO_2 groups; that is, their thermodynamically most stable form in unhindered compounds in solution and heterogeneity due to twisting in dinitrophenyl haptens would have to involve regions of the molecule other than the nitro groups.

Dinitrophenyl Residue. The fact that the 2- and 4-nitro groups suffer very similar perturbation in all haptens suggests that the corresponding regions of the antibody site consist of the same amino acid residues in an almost identical three-dimensional array. Alternatively, any substitutions are such as not to affect interactions which occur with the nitro groups. The nature and orientation of these amino acid residues constitute one of the central problems of immunochemistry. The present study does not identify these amino acids but does suggest that they are arranged to provide a particular sort of array of charges or dipoles. The site is viewed as being more than a simple hydrophobic slot shaped to accommodate the haptens. Hydrophobic interactions may well be involved, but they are not the whole story. Tryptophan has been suggested to be in the site (Little and Eisen, 1967) and its indole residue could provide some of the positive charge necessary. Calculations of partial charges on indole residue atoms show there is a net positive charge on the nitrogen (Pullman and Pullman, 1963).

General Discussion. In summary, resonance Raman spectroscopy has been shown to be a useful technique for investigating hapten–antibody interactions. Changes in hapten structure induced by binding could be detected and information directly related to antibody site structure could be obtained. It is expected that subsequent applications will provide much useful information about the complex and the structure of the site.

In evaluating the technique it is noteworthy that the present study has been able to extract only a fraction of the information potentially available. One reason is that the system used, although of great interest to immunochemists, is not ideal by spectroscopic standards. It was chosen because of the absence of appropriate guidelines and also simply because it was available. It was not optimal because the NO_2 group frequency which could be employed is not a good group frequency in the sense of being sensitive to small perturbations. Also, the substituents of the naphthyl ring do not, with the possible exception of C–O, have well-defined group frequencies which are resonance enhanced. In contrast, the azo group has an excellent group frequency. As a result, only minimal amounts of information were obtained about the NO_2 group and phenolic and sulfonate ions. More information could be expected by using haptens with substituents having better group frequencies. Possible candidates, which at the same time could also be part of a chromophoric π system, and hence yield resonance Raman spectra are the alkyne, cyano, carboxyl, carboxyl ester, and azido groups.

Another factor limiting the amount of information which could be obtained is that only a fraction of the available Raman data could be interpreted easily. This stemmed partly from the complexity of the haptens used, the poor group frequencies of the substituents, and the occurrence of fluorescence on binding of Dnp-lysine or in the solid state. However, the major factor is that it is difficult in general to interpret Raman spectra completely. One solution would be to use simpler haptens bearing substituents with well-defined group frequencies.

The occurrence of fluorescence, although a difficulty in the present study, is essentially a technical problem and is being overcome through the development of gated spectrometer detection coupled with pulsed lasers which permit determination of Raman spectra before fluorescence builds up.

An additional factor limiting the amount of information which could be extracted is that ancillary information was required; crystal-structure data coupled with Raman spectra of these crystals would be necessary to be definitive about several points. Again, judicious choice of haptens would go a long way in facilitating interpretation and providing additional information. For instance, the use of haptens whose crystal structures were known could be of obvious help.

A point of interest regarding the technique is that it is capable of answering the question as to whether or not the structure of a particular binding site on a protein is the same in the crystalline state as in solution. For antibodies the binding site could be the hapten binding site. Since resonance Raman spectra can be obtained with solids as well as liquids, the question can be answered by comparing spectra of a suitable colored ligand bound to the relevant site in solution and in the crystal. Identity of spectra indicates with a high degree of probability the site structures would also be identical.

A feature of the technique is that only small volumes of solution are required. Most of the spectra reported here were obtained on 5-μl samples. This advantage must be balanced against the need for using multiple samples in repeating experiments to ensure that the spectra do not contain artifacts. However, as sample handling techniques and spectrometer technology in general improve, the need for these multiple samples may decrease.

The need for only small samples will be of importance in designing future experiments. When studies are carried out where the hapten used for immunization is modified, say by substitution of a carboxyl for a nitro group, the combining constant will decrease; the fraction of unbound haptens will therefore decrease and possibly introduce complications in interpretation. However, this can be corrected by increasing antibody concentration. Since only small volumes are required, this concentration can be carried out without requiring inordinate amounts of antibody.

Acknowledgments

We would like to thank Dr. H. J. Bernstein for use of the Spex spectrometer and Mr. R. Carriere for excellent technical assistance.

References

Abrahams, S. C. (1950), *Acta Crystallogr. 3*, 194.
Behringer, J. (1967a), *Raman Spectrosc. 1*, 168.
Behringer, J. (1967b), *Raman Spectrosc. 1*, 205.
Bellamy, L. J. (1958a), The Infrared Spectra of Complex Molecules, London, Methuen.
Bellamy, L. J. (1958b), The Infrared Spectra of Complex Molecules, London, Methuen, p 303.
Bellamy, L. J. (1968), Advances in Infrared Group Frequencies, London, Methuen, pp 230–231.
Bist, H. D., Brand, J. C. D., and Williams, D. R. (1967), *J. Mol. Spectrosc. 24*, 402.
Brunner, H., Mayer, A., and Sussner, H. (1972), *J. Mol. Biol. 70*, 153.
Carey, P. R., Schneider, H., and Bernstein, H. J. (1972), *Biochem. Biophys. Res. Commun. 47*, 588.
Conduit, C. P. (1959), *J. Chem. Soc.*, 3273.
Corruccini, R. J., and Gilbert, E. C. (1939), *J. Amer. Chem. Soc. 61*, 2925.

Eisen, H. N., Belman, S., and Carsten, M. F. (1953), *J. Amer. Chem. Soc. 75*, 4583.
Eisen, H. N., Simms, E. S., Little, J. R., and Steiner, L. A. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 559.
Froese, A. (1968), *Immunochemistry 5*, 253.
Froese, A., Goldwater, S. G., and Sehon, A. H. (1969), *Protides Biol. Fluids, Proc. Colloq. 16*, 167.
Grant, D. F., and Richards, J. P. (1969), *Acta Crystallogr., Sect. B 25*, 564.
Green, J. H. S., Harrison, D. J., and Kynasten, W. (1971), *Spectrochim. Acta, Part A 27*, 2199.
Hacker, H. (1965), *Spectrochim. Acta 21*, 1989.
Hartley, G. S. (1938), *J. Chem. Soc.*, 633.
Hsia, J. C., and Little, J. R. (1971), *Biochemistry 10*, 3742.
Kelly, K. A., Sehon, A. H., and Froese, A. (1971), *Immunochemistry 8*, 613.
Kiefer, W., and Bernstein, H. J. (1971), *Appl. Spectrosc. 25*, 609.
Kübler, R., Lüttke, W., and Weckherlin, S. (1960), *Z. Elektrochem. 64*, 5.
Larkindale, J. P., and Simkin, D. J. (1972), *Spectrochim. Acta, Part A 28*, 485.
Leite, R. C. C., Porto, S. P. S., and Damen, T. C. (1967), *Appl. Phys. Lett. 10*, 100.
Liberti, P. A., Stylos, W. A., and Maurer, P. H. (1972a), *Biochemistry 11*, 3312.
Liberti, P. A., Stylos, W. A., Maurer, P. H., and Callahan, H. J. (1972b), *Biochemistry 11*, 3321.
Little, J. R., and Eisen, H. N. (1967), *Biochemistry 6*, 3119.
Marrack, J. H., Hoch, H., and Johns, R. G. S. (1951), *Brit. J. Exp. Pathol. 32*, 212.
Metzger, H., Wofsy, L., and Singer, S. J. (1963), *Arch. Biochim. Biophys. 103*, 206.
Oster, G., and Nishijima, Y. (1956), *J. Amer. Chem. Soc. 78*, 1581.
Pinchas, S. (1972), *Spectrochim. Acta, Part A 28*, 801.
Pinchas, S., Samuel, D., and Silver, B. L. (1964), *Spectrochim. Acta 20*, 179.
Poljack, R. J., Amzel, L. M., Avey, H. P., Becka, L. N., and Nisonoff, A. (1972), *Nature (London), New Biol. 235*, 137.
Pressman, D., and Grossberg, A. L. (1968), The Structural Basis of Antibody Specificity, New York, N. Y., W. A. Benjamin.
Pullman, B., and Pullman, A. (1963), Quantum Biochemistry, New York, N. Y., Interscience.
Ray, A., and Cebra, J. J. (1972), *Biochemistry 11*, 3647.
Robbins, J. B., Haimovich, J., and Sela, M. (1967), *Immunochemistry 4*, 11.
Rockey, J. H., Montgomery, P. C., Underdown, B. J., and Dorrington, K. J. (1972), *Biochemistry 11*, 3172.
Scheraga, H. A. (1968), *Advan. Phys. Org. Chem. 6*, 103.
Scott, R. A., and Scheraga, H. A. (1965), *J. Chem. Phys. 42*, 2209.
Seff, K., and Trueblood, K. N. (1968), *Acta Crystallogr., Sect. B 24*, 1406.
Shorygin, P. P., and Il'icheva, Z. F. (1958), *Bull. Acad. Sci. USSR, Phys. Ser. 22*, 1046.
Strausbauch, P. H., Weinstein, Y., Wilcheck, M., Shaltiel, S., and Givol, D. (1971), *Biochemistry 10*, 4342.
Strekas, T. C., and Spiro, T. G. (1972), *Biochim. Biophys. Acta 278*, 188.
Trotter, J. (1959), *Can. J. Chem. 37*, 905.
Urbanski, T., and Dabrowska, U. (1959), *Bull. Acad. Polon. Sci. 7*, 235.
Utsumi, S., and Karush, F. (1964), *Biochemistry 3*, 1329.
Van Veen, A., Verkade, P. E., and Wepster, B. M. (1957), *Recl. Trav. Chim. Pays-Bas, 76*, 801.
Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1470.
Winkler, M. (1962), *J. Mol. Biol. 4*, 118.
Yan, J. F., Vanderkooi, G., and Scheraga, H. A. (1968), *J. Chem. Phys. 49*, 2713.