STUDIES ON THE EFFECT OF THE CARRIER MOLECULE ON ANTIHAPTEN ANTIBODY SYNTHESIS*

II. Carrier Specificity of Anti-2,4-Dinitrophenyl-Poly-L-Lysine Antibodies

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(Received for publication 23 December 1965)

Certain properties of guinea pig antihapten antibodies were shown in a companion paper (1) to be affected by the nature of the carrier molecule used for immunization; these were the average intrinsic association constant (K_0) with hapten, the serum antibody concentration, and the relative amounts of γ_1- and γ_2-antihapten antibody produced by hyperimmunized animals.

Another effect of the carrier molecule, whose importance is becoming increasingly recognized, is its contribution to immunological specificity. Marked "carrier" specificity has been observed with hapten-protein systems in delayed hypersensitivity reactions (2, 3) and in the early secondary response (4). These reactions are generally believed to be mediated by antibody which is presumed to be cell bound. Such observations suggested that antihapten antibodies themselves should exhibit a similar carrier specificity under appropriate experimental conditions. However, since many antigen-antibody reactions (e.g., precipitation, anaphylaxis, and Arthus reactions) require relatively little energy, the contribution of the hapten alone was found, in most cases, to be sufficient (3, 5). As an awareness of the possible contribution of the carrier in immunological reactions grew, carrier specificity of antihapten antibodies has been observed in several systems: passive cutaneous anaphylaxis in the guinea pig with early immunization sera (6), wheal and flare reactions in humans (7), and quantitative precipitin reactions with certain selected systems (8–10).

Studies utilizing precipitation have demonstrated the existence of an antibody population which can be precipitated only by the immunizing hapten-protein complex (8, 9) and not by the carrier protein or the hapten conjugated to heterologous pro-

* This study was supported by the United States Public Health Service Research Grants 5-RO1-AMO 8805-02 and AI-2094, and the Health Research Council of the City of New York under Contracts I-138 and I-464.

Computor facilities of the Department of Neurology, Section on Communications Sciences supported by United States Public Health Service Research Grant FR-00002 were made use of during the course of this work.

‡ Recipient of a Public Health Service fellowship No. 2-F3-AI,22,420-02 from the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

teins. In the past these results have been interpreted as indicating the existence of new determinants, referred to as "link" determinants, created by the apposition of hapten and protein. However, as suggested by the carrier specificity of antihapten antibodies isolated by elution with specific hapten, a more appropriate interpretation would be that the so-called link determinant antibodies have an immunological specificity equally involving the hapten and the carrier.

In spite of the numerous reports of carrier specificity no quantitative measurements have been made of the energy contributed by the specific interaction of the antihapten antibody with the carrier molecule. The reaction of guinea pig anti-2,4-dinitrophenyl-poly-L-lysine antibodies (DNP-PLL) with a lightly substituted DNP-PLL antigen offers considerable promise for measurements of carrier specificity because: (*a*) it can be expected that the environment of all DNP groups would be very similar in all PLL molecules, and (*b*) the affinity of anti-DNP-PLL and of anti-DNP-protein antibodies for lightly substituted DNP-PLL molecules could be measured by fluorescence quenching, in spite of the large size of the ligand, and compared with their respective affinities for ε-DNP-L-lysine. In this system it is possible therefore to obtain an estimate of the magnitude of the forces involved in carrier specificity and some information with regard to their nature.

Materials and Methods

Preparation of DNP Derivatives of Proteins and Polylysines, Immunization of Animals, Precipitin Curves, and Purification of Anti-DNP Antibodies.—Bovine gamma globulin (BGG), bovine serum albumin (BSA), hen ovalbumin (Ova), calf skin gelatin (Gel), guinea pig serum albumin (GPA), bovine fibrinogen (BF), and poly-L-lysine (PLL) were purchased or prepared as described in the preceding paper (1). Poly-D-lysine (PDL) of 109 residues/molecule was purchased from Pilot Chemicals Inc., Watertown, Massachusetts.

DNP derivatives of proteins and polylysines were prepared and characterized as indicated in the previous paper (1). The following compounds were prepared: DNP_{169}-BF, DNP_{62}-BGG, DNP_{70}-BGG, DNP_{46}-BSA, DNP_{52}-BSA, DNP_{19}-Ova, DNP_{48}-Gel (per 100,000 assumed molecular weight), DNP_{40}-GPA, $DNP_{0.06}$-PLL_{240}, $DNP_{0.1}$-PLL_{240}, $DNP_{0.6}$-PLL_{240}, DNP_1-PLL_{240}, DNP_2-PLL_{240}, DNP_7- PLL_{240}, DNP_{11}-PLL_{240}, DNP_{32}-PLL_{1420}, DNP_{44}-PLL_{1420}, and $DNP_{0.77}$-PDL_{109}. In each instance subscripts refer to average number of DNP groups and in the case of polylysines to the number of lysines per molecule.

Hartley strain guinea pigs were immunized using complete Freund's adjuvant as described in the companion paper (1). The following compounds were used for immunization: DNP_{62}-BGG, DNP_{70}-BGG, DNP_{46}-BSA, DNP_{52}-BSA, DNP_{19}-Ova, DNP_{48}-Gel, DNP_{40}-GPA, DNP_{11}-PLL_{240}, DNP_{32}-PLL_{1420}, and DNP_{44}-PLL_{1420}.

Precipitin curves were carried out according to the methods of Eisen et al. (9) as summarized previously (1).

Antibodies with DNP specificity were purified as described by Farah et al. (5) by elution from specific precipitates, formed at equivalence with DNP-BF, with 0.1 M 2,4-dinitrophenol. Further procedural details and characterization of the purified antibodies are presented in the companion paper (1).

All reagents used were the same as described in the preceding paper (1).

Preparation of Fab' Fragments.—Fab' fragments (univalent fragments obtained by mild

reduction of pepsin digests of guinea pig antibody) were prepared as described by Nisonoff (11). Purified anti-DNP antibody at a concentration of 5 to 10 mg/ml was dialyzed overnight against 0.1 M sodium acetate. The pH was then adjusted to 4.0 with 1 N acetic acid. Thrice recrystallized pepsin (Pentex, Inc., Kankakee, Illinois) was added in an enzyme-substrate weight ratio of 1:50. Digestion was allowed to proceed at 37°C for 8 hr. Following digestion the sample was clarified by centrifugation and brought to pH 7.5 by the addition of 1 N NaOH. Digested antibody was precipitated at 18% Na_2SO_4, collected by centrifugation, washed once with 18% Na_2SO_4, dissolved in 0.1 N sodium acetate, and dialyzed overnight against 0.1 N sodium acetate. The pH was then brought to 4.5 with 1 N acetic acid and the solution made 0.015 M in 2-mercaptoethanol. The reaction mixture was incubated, under N_2, for 90 min at 37°C and then made 0.03 M in iodoacetamide. The final preparation was then extensively dialyzed against 0.15 M NaCl, 0.01 M PO_4, pH 7.6 (PBS).

Measurement of Antibody Binding by Fluorescence Quenching.—The measurements were carried out as described in the preceding paper (1). All titrations were performed at 26°C on duplicate 2.0 ml samples of 25 to 30 μg antibody/ml in PBS. In the case of titrations with DNP-PLL conjugates, the amounts of ligand added were measured as DNP. Antibody concentrations were calculated from the absorbancy at 278 mμ using $E_{1cm}^{1\%} = 13.2$ for guinea pig antibody and 15.4 for the Fab' fragment (12). Average intrinsic association constants (K_0) were calculated from fluorescence-quenching measurements (13, 14) using 100% as the value for maximum quenching as justified in the previous paper (1). Standard free energy (ΔF_0) was calculated from the relationship $\Delta F_0 = -RT \ln K_0$, where R = the gas constant and T = absolute temperature.

Selection of an Appropriate DNP-PLL Conjugate for Equilibrium Measurements of Interaction with anti-DNP Antibodies.—It is apparent that equilibrium determinations for the interactions of antigen and anti-DNP antibodies cannot be made with the relatively highly conjugated DNP-PLL antigens used for immunization since the formation of insoluble aggregates would render such measurements thermodynamically uninterpretable. A lightly substituted DNP-PLL which would not precipitate with antibody was required for these measurements. Ideally the conjugate to be used should be as lightly substituted as possible and still behave as a reasonable model for the more highly conjugated immunizing antigen. Several DNP-PLL preparations were studied: $DNP_{0.06}$-PLL_{240}, $DNP_{0.1}$-PLL_{240}, $DNP_{0.6}$-PLL_{240}, DNP_1-PLL_{240}, and DNP_2-PLL_{240}. Fluorescence-quenching determinations with these preparations and a standard anti-DNP-PLL antibody pool showed that the native fluorescence of the antibody was more effectively quenched by $DNP_{0.6}$-PLL_{240} than by $DNP_{0.1}$-PLL_{240}, or $DNP_{0.06}$-PLL_{240}. Titrations yielded identical fluorescence-quenching curves with DNP_1-PLL_{240}, DNP_2-PLL_{240}, and with $DNP_{0.6}$-PLL_{240}. PLL_{240} alone caused no quenching of anti-DNP antibody fluorescence and $DNP_{0.6}$-PLL_{240} caused no quenching of fluorescence of normal guinea pig γ_2-globulin.

The quenching behavior of very lightly substituted DNP-PLL conjugates could be correlated with the spectral properties of $DNP_{0.1}$-PLL_{240} as compared to ϵ-DNP-L-lysine, to $DNP_{0.6}$-PLL_{240}, or to more highly conjugated DNP-PLL compounds. ϵ-DNP-L-lysine has an absorption maximum at 360 mμ and a minimum at 300 mμ (ϵ 360 mμ/ϵ 300 mμ = 7.4). $DNP_{0.1}$-PLL_{240} has a similar absorbancy at 360 mμ but an increased absorbancy at 300 mμ (ϵ 360 mμ/ϵ 300 mμ = 1.6). $DNP_{0.06}$-PLL_{240} has spectral properties similar to $DNP_{0.1}$-PLL_{240}. (These values take into account the slight absorbancy of PLL at 300 mμ). In contrast, $DNP_{0.6}$-PLL_{240} and all more highly conjugated DNP-PLL's (the most highly conjugated compound studied was DNP_{39}-PLL_{240}) exhibit ϵ 360 mμ/ϵ 300 mμ ratios similar to that of ϵ-DNP-L-lysine (approximately 6). It appears therefore that the structure of the determinant in DNP-PLL_{240} molecules with 0.6 or higher average numbers of groups per molecule is different from the structure of the determinant in the more lightly conjugated homopolymers. From the spectral data and the behavior in fluorescence quenching discussed above one can assume

that the environment of the DNP group in $DNP_{0.6}$-PLL_{240} is similar to that present in the more highly substituted immunizing antigens.

Although the spectral differences between $DNP_{0.1}$-PLL_{240} and $DNP_{0.6}$-PLL_{240} cannot be explained critically it is probable that they are related to some interaction between the charged ϵ-amino groups of unsubstituted lysines and nitro groups on the aromatic DNP ring. This interaction might occur to a greater extent in the monosubstituted PLL molecules which predominate in the $DNP_{0.1}$-PLL_{240} preparation. The existence, with high frequency, of two or more DNP groups per PLL molecule in $DNP_{0.6}$-PLL_{240} and higher conjugates presumably leads to the type of spectral and immunochemical behavior exhibited by these molecules. Indeed based on the assumption of random conjugation of DNP to PLL molecules one would expect that in $DNP_{0.6}$-PLL_{240}, 45% of DNP groups would be present on PLL molecules

TABLE I

Delayed Reactivity of "Responder" Guinea Pigs Immunized with DNP_{11}-PLL_{240}

Animal No.	Skin tested with		
	10 μg DNP_{11}-PLL_{240}	0.55 μg DNP_{11}-PLL_{240}	10 μg $DNP_{0.6}$-PLL_{240}
	mm	*mm*	*mm*
1	11	5	10
2	12	5	9
3	10	4	10
4	11	3	10
5	8	3	6
6	12	4	10
7	12	5	11
8	10	4	10
9	14	4	10
10	10	3	9

Hartley strain guinea pigs were immunized by foot-pad injection of 100 μg DNP_{11}-PLL_{240} emulsified in complete Freund's adjuvant. 10 days later they were given intradermal injections of 0.1 ml of the antigens listed in the table above. Results are recorded as average diameter of induration.

bearing two or more DNP groups. Considering the known interactions of DNP groups, conjugation is probably not completely random and the true value is probably considerably higher which is consistent with the identical spectral and immunological behavior of $DNP_{0.6}$-PLL_{240} and more highly substituted conjugates.

Because of the considerations discussed above, $DNP_{0.6}$-PLL_{240} was selected for use throughout the remainder of these studies. Two additional experiments were carried out to further justify this choice of ligand. (*a*) Table I shows that $DNP_{0.6}$-PLL_{240} was almost as efficient as DNP_{11}-PLL_{240} in eliciting delayed hypersensitivity reactions in guinea pigs sensitized by the latter compound when given in equal amounts with regard to PLL and was considerably more efficient when given in equal amounts with regard to DNP groups. (*b*) Considering the high frequency in $DNP_{0.6}$-PLL_{240} of DNP groups on PLL molecules bearing two or more DNP groups, evidence must be presented that the antigenic determinants when such molecules are used as ligands, and the specificity of the anti-DNP antibodies studied, involved only a single DNP group rather than a complex structure formed from the interaction of 2 or more DNP

groups. Calculations of antibody valence (n) assuming a single DNP group per antigenic determinant were made for various anti-DNP antibodies from fluorescence quenching curves with $DNP_{0.6}$-PLL_{240} and with ϵ-DNP-L-lysine. If more than one DNP was involved per determinant, then such calculations would lead to a value for n distinctly greater than 2. Extrapolation of the initial linear portion of the curves for quenching of antibody fluorescence (see Fig. 1) to their intersection with the abscissa [Q_{max} = 100%; see previous paper (1)] provides an estimate of the number of antibody sites present (13). Values of n, calculated from such data are presented in Table II. It is apparent that the same value for n was obtained with $DNP_{0.6}$-PLL_{240} and with the univalent hapten ϵ-DNP-L-lysine. In all cases n approached the expected value of 2 (15).

TABLE II

Valence (n) of Guinea Pig Anti-DNP Antibodies Calculated from Fluorescence Titrations

Antibody	Ligand = $DNP_{0.6}$- PLL_{240}	Ligand = ϵ-DNP -L-Lysine
Anti-DNP-PLL		
67-9	1.8	1.8
71-1	1.7	2.2
8	1.5	1.8
III-2	1.5	1.6
Anti-DNP-BGG		
22	1.7	1.6
28	1.8	2.1
29	2.0	1.7
Anti-DNP-Ova		
17	1.7	1.8
67	2.0	2.3

RESULTS

Measurement of Energy Involved in "Carrier" Specificity of Anti-DNP-PLL Antibodies.—Fig. 1 compares the effect of ϵ-DNP-L-lysine and of $DNP_{0.6}$-PLL_{240} on the native fluorescence of purified guinea pig anti-DNP-PLL antibody. Greater quenching of fluorescence was clearly achieved with $DNP_{0.6}$-PLL_{240} than with the simple hapten demonstrating a higher affinity of the antibody for the polylysine conjugate as compared with ϵ-DNP-L-lysine. Furthermore, the $DNP_{0.6}$-PLL_{240} curve had a greater slope than the ϵ-DNP-L-lysine curve at all corresponding ligand concentrations showing that all of the antibody molecules that were titrated possessed higher affinity for $DNP_{0.6}$-PLL_{240} than for ϵ-DNP-L-lysine. Quenching of the fluorescence of purified anti-DNP-Ova antibodies with the same ligands yielded strikingly different results. Considerably less quenching of anti-DNP-Ova was obtained with $DNP_{0.6}$-PLL_{240} than with ϵ-DNP-L-lysine (Fig. 1). Equilibrium constants (K_0) calculated

from the data obtained from titrations with both ligands of anti-DNP antibodies purified from sera of animals immunized with DNP conjugated to a variety of different carriers are presented in Table III. Purified anti-DNP-PLL antibodies prepared from the sera of 6 individual guinea pigs and from

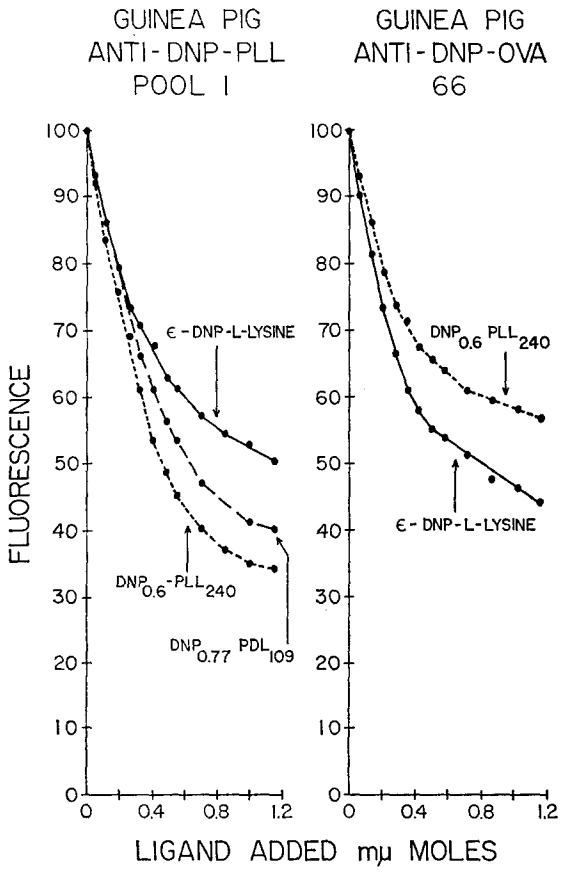

FIG. 1. Fluorescence quenching of anti-DNP antibodies by various DNP ligands. Measurements were carried out on 2 ml samples containing 30 μg purified antibody/ml in PBS at 26°C. Amounts of ligands added are expressed in terms of DNP groups.

two pools of anti-DNP-PLL sera exhibited consistently higher K_0's (3.6 to 360 times greater) when titrated with $DNP_{0.6}$-PLL_{240} than with ϵ-DNP-L-lysine.

The standard free energies (ΔF^0) of the antibody ligand interactions were calculated from the equilibrium constants determined above (Table III). The difference in free energy between the reaction of antibody with $DNP_{0.6}$-PLL_{240} and with ϵ-DNP-L-lysine (ΔF^0 for $DNP_{0.6}$-PLL_{240} $-\Delta F^0$ for ϵ-DNP-L-lysine)

TABLE III

Interaction of Anti-DNP Antibodies with ε-DNP-L-Lysine and with $DNP_{0.6}$-PLL_{240}

Immunizing carrier	Identification No.	K_0 With $DNP_{0.6}$-PLL_{240}	K_0 With ε-DNP-L-lysine	ΔF_1^0 $DNP_{0.6}$-PLL_{240}	ΔF_2^0 ε-DNP-L-lysine	ΔF_1^0-ΔF_2^0 $\Delta(\Delta F^0)$
		liters/mole of sites $\times 10^{-6}$	*liters/mole of sites* $\times 10^{-6}$	*kcal/mole*	*kcal/mole*	*kcal/mole*
PLL	V-3	83.	2.3	−10.82	−8.71	−2.11
	III-2	19.	4.7	−9.98	−9.13	−0.85
	7	52.	4.0	−10.58	−9.04	−1.54
	67-9	17.	3.0	−9.91	−8.89	−1.02
	8	50.	6.1	−10.52	−9.30	−1.22
	71-1	1.8	0.49	−8.57	−7.80	−0.77
	Pool 1	24.	3.2	−10.10	−8.92	−1.28
	Pool 6	13.	1.3	−9.74	−8.37	−1.37
					Mean	−1.27
BGG	Pool 1	0.63	5.4	−7.94	−9.21	+1.28
	22	1.0	4.4	−8.23	−9.10	+0.87
	27	0.57	1.3	−7.89	−8.37	+0.48
	28	1.5	2.2	−8.48	−8.69	+0.21
	29	0.68	2.7	−8.00	−8.83	+0.83
	60	0.90	5.5	−8.15	−9.23	+1.08
	62	0.23	2.0	−7.34	−8.63	+1.29
	64	0.37	5.7	−7.63	−9.26	+1.63
					Mean	+0.96
BSA	33	0.042	0.44	−6.38	−7.72	+1.39
	75	0.059	0.80	−6.52	−8.09	+1.57
	77	0.063	0.63	−6.58	−7.95	+1.37
	78	0.045	0.55	−6.38	−7.86	+1.48
	79	0.0047	0.35	−5.22	−7.60	+2.38
					Mean	+1.64
Ova	17	3.2	2.5	−8.91	−8.76	−0.15
	65	1.3	6.7	−8.37	−9.36	+0.99
	66	1.6	8.2	−8.50	−9.47	+0.97
	67	0.32	0.70	−7.56	−8.02	+0.46
	68	1.2	2.7	−8.34	−8.81	+0.47
					Mean	+0.55
GPA	69	0.063	0.50	−6.58	−7.82	+1.24
Gel	20	0.060	0.48	−6.56	−7.79	+1.23
	22	15.	5.3	−9.85	−9.20	−0.65
	70	0.048	0.51	−6.42	−7.83	+1.41
	72	0.38	0.86	−7.65	−8.13	+0.48
	73	1.0	0.45	−8.23	−7.74	−0.49
	74	0.29	0.55	−7.48	−7.86	+0.38
					Mean	+0.43

* Measurements carried out in PBS at 26°C. Both ligands added in terms of DNP groups

referred to hereafter as $\Delta(\Delta F^0)$ was negative for every preparation of anti-DNP-PLL studied. That is, antibody made using PLL as carrier bound $DNP_{0.6}$-PLL_{240} more strongly than it bound the simple hapten by an average of 1.27 kcal/mole (Table III). In contrast, K_0's with $DNP_{0.6}$-PLL_{240} were consistently lower than those with ϵ-DNP-L-lysine when anti-DNP-BGG, anti-DNP-BSA, anti-DNP-Ova, and anti-DNP-GPA were titrated. In each of these cases, the $\Delta(\Delta F^0)$ was positive and was significantly different from that obtained with anti-DNP-PLL [$P < .01$ by t test (16)]. Furthermore, $\Delta(\Delta F^0)$ for anti-DNP-BSA was significantly different from that for anti-DNP-Ova ($P < .01$) and from that for anti-DNP-BGG ($P < .05$).

Six guinea pig anti-DNP-Gel antibodies were also titrated with both ligands. Four bound $DNP_{0.6}$-PLL_{240} less strongly than ϵ-DNP-L-lysine while two exhibited the opposite behavior. Even in the latter two cases the $\Delta(\Delta F^0)$ was less negative than any of the values obtained for anti-DNP-PLL antibodies (Table III).

It thus appeared that the PLL molecule contributed between 0.8 and 2.1 kcal/mole to the interaction with anti-DNP-PLL; and, in addition, the presence of the PLL molecule diminished the energy of interaction of the DNP group with anti-DNP-protein antibodies by up to 2.4 kcal/mole.

Since $DNP_{0.6}$-PLL_{240} is a highly charged molecule, the positive values for $\Delta(\Delta F^0)$ (Table III) observed with anti-DNP-protein antibodies might be due to electrostatic repulsive forces. In order to explore this possibility binding measurements were carried out in 1.50 M NaCl, 0.01 M PO_4, pH 7.6 (1.50 M PBS) as well as in our usual solvent, 0.15 M NaCl, 0.01 M PO_4, pH 7.6 (0.15 M PBS). Titration of anti-DNP-PLL antibody in 1.50 M PBS with $DNP_{0.6}$-PLL_{240} and with ϵ-DNP-L-lysine yielded curves similar to those obtained in 0.15 M PBS (Fig. 2). On the other hand, the poorer quenching of anti-DNP-BGG antibodies by $DNP_{0.6}$-PLL_{240} than by ϵ-DNP-L-lysine observed in 0.15 M PBS was virtually abolished by carrying out such measurements in 1.50 M PBS. The K_0 for the interaction of anti-DNP-BGG with ϵ-DNP-L-lysine was little different in 1.50 M PBS from that in 0.15 M PBS. However, K_0 of interaction of anti-DNP-BGG with $DNP_{0.6}$-PLL_{240} was considerably higher in 1.50 M PBS than in 0.15 M PBS. The $\Delta(\Delta F^0)$ changed from +0.47 and +0.82 kcal/mole for two individual anti-DNP-BGG antibodies in 0.15 M PBS to +0.02 and +0.11 kcal/mole respectively when measured in 1.50 M PBS. Thus, electrostatic repulsion seems to explain a considerable portion of the observed diminution of energy of interaction when anti-DNP-BGG antibodies are titrated with $DNP_{0.6}$-PLL_{240}.

Since $DNP_{0.6}$-PLL_{240} represents a population of molecules of different integral number of DNP groups per PLL molecule and since many of the DNP groups are believed to be on PLL molecules containing more than one DNP group, the possibility exists that both binding sites of an antibody molecule

might bind to DNP groups on the same PLL molecule. Such binding, for reasons discussed subsequently, would be expected to result in greater strength

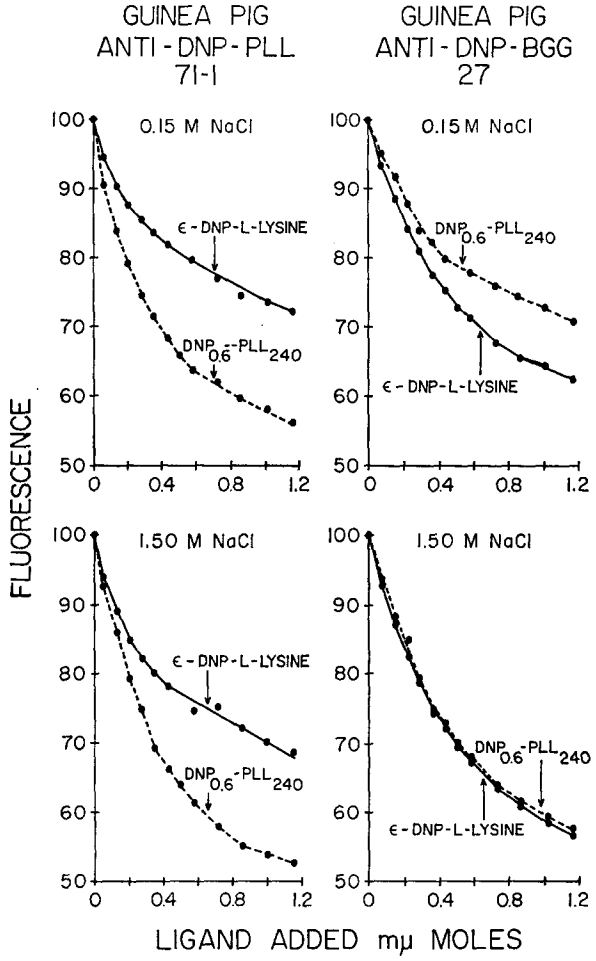

FIG. 2. The effect of salt concentration on interaction of anti-DNP antibodies with DNP ligands. The left panels present results with guinea pig anti-DNP-PLL 71-1 and the right panels present results with guinea pig anti-DNP-BGG 27. Measurements were carried out on 2 ml samples containing 30 μg purified antibody/ml in PBS at 26°C. Amounts of ligands added are expressed in terms of DNP groups.

for each of the bonds formed as compared with binding to determinants on different antigen molecules. That is, a type of "cooperative interaction" between divalent antibody and divalent or multivalent antigen might exist which would augment the binding energy. Such cooperative effects could be measured

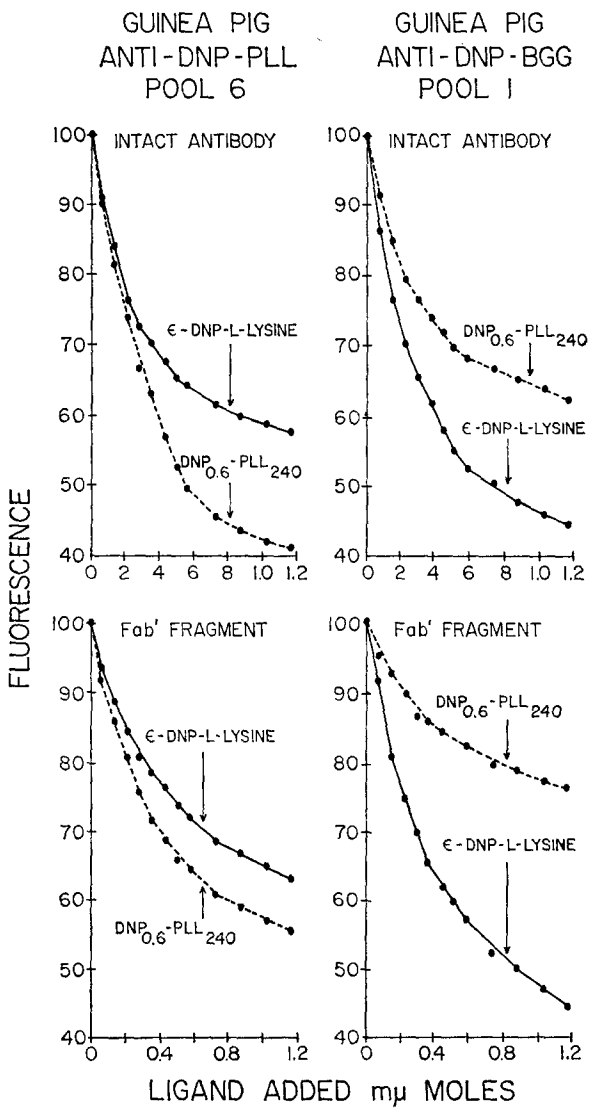

FIG. 3. Fluorescence quenching of intact anti-DNP antibodies and their univalent fragments (Fab') by DNP ligands. The left panels present results with guinea pig anti-DNP-PLL pool 6 and the right panels with guinea pig anti-DNP-BGG pool 1. Measurements were carried out on 2 ml samples in PBS at 26°C. The concentration of purified intact antibody was 30 μg/ml while that of Fab' fragments was 26 μg/ml. Amounts of ligands added are expressed in terms of DNP groups.

by comparing intact antibody and univalent antibody fragment (Fab') with regard to their binding to $DNP_{0.6}$-PLL_{240}. From Fig. 3 it can be seen that intact antibodies and Fab' fragments of both anti-DNP-PLL and anti-DNP-BGG had similar fluorescence quenching titration curves with ε-DNP-L-lysine. In both cases calculated K_0's with ε-DNP-L-lysine were essentially identical for intact antibody and for Fab' fragments (Table IV). In contrast, fluorescence quenching titration curves of anti-DNP-PLL and anti-DNP-BGG Fab' fragments with $DNP_{0.6}$-PLL_{240} revealed less quenching than was displayed with the corresponding intact antibodies. $\Delta(\Delta F^0)$ for anti-DNP-PLL was −1.38 kcal/mole for intact antibody and −0.41 kcal/mole for Fab' fragments.

TABLE IV

*Interaction of intact Guinea Pig Anti-DNP Antibody and Fab' Fragments with ε-DNP-L-Lysine and with $DNP_{0.6}$-PLL_{240}**

Antibody preparation	Solvent	K_0 with $DNP_{0.6}$-PLL_{240}	K_0 with ε-DNP-L-Lysine	ΔF^0_1 $DNP_{0.6}$-PLL_{240}	ΔF^0_2 ε-DNP-L-Lysine	$\Delta F^0_1 - \Delta F^0_2$ $\Delta(\Delta F^0)$
	molarity PBS	*× 10^{-6} liters/mole*	*× 10^{-6} liters/mole*	*kcal/M*	*kcal/M*	*kcal/M*
Anti-DNP-PLL pool 6 intact	0.15	13.	1.3	−9.75	−8.37	−1.38
Anti-DNP-PLL pool 6 Fab' fragment	0.15	2.2	1.1	−8.69	−8.28	−0.41
Anti-DNP-BGG pool 1 intact	0.15	0.75	7.9	−8.06	−9.45	+1.39
Anti-DNP-BGG pool 1 Fab' fragment	0.15	0.29	8.0	−7.49	−9.45	+1.96
Anti-DNP-BGG pool 1 Fab' fragment	1.50	1.0	5.8	−8.22	−9.26	+1.04

* Reactions carried out at 26°C. Both ligands added in terms of DNP groups.

For anti-DNP-BGG intact antibody, $\Delta(\Delta F^0)$ was +1.39 kcal/mole while for the corresponding Fab' fragments, $\Delta(\Delta F^0)$ was +1.96 kcal/mole. Thus a cooperative effect is observed when intact antibody is used of a magnitude of −0.6 to −1.0 kcal/mole of sites.

Titrations of anti-DNP-BGG Fab' fragments in 1.50 M PBS with ε-DNP-L-lysine and $DNP_{0.6}$-PLL_{240} revealed that $\Delta(\Delta F^0)$ was +1.04 kcal/mole, whereas it was +1.96 kcal/mole in 0.15 M PBS. Thus, electrostatic repulsion apparently does not account for all of the diminished energy of interaction of anti-DNP-BGG with $DNP_{0.6}$-PLL_{240} as compared to its interaction with ε-DNP-L-lysine.

Demonstration of Carrier Specificity in Precipitin Reactions with Anti-DNP-Protein Antibodies.—In Fig. 4 *a*, the precipitation of purified guinea pig anti-DNP-Ova antibody of the γ_1-immunoglobulin class by DNP-Ova is compared with the precipitation of purified γ_1-anti-DNP-BGG and γ_1-anti-DNP-Gel

by the same antigen. Similar maximum percentages of anti-DNP-Ova and anti-DNP-BGG were precipitated and only slightly less anti-DNP-Gel. However, in antigen excess, DNP-Ova formed soluble complexes with anti-DNP-Ova more readily than with the other antibodies tested. The antibodies

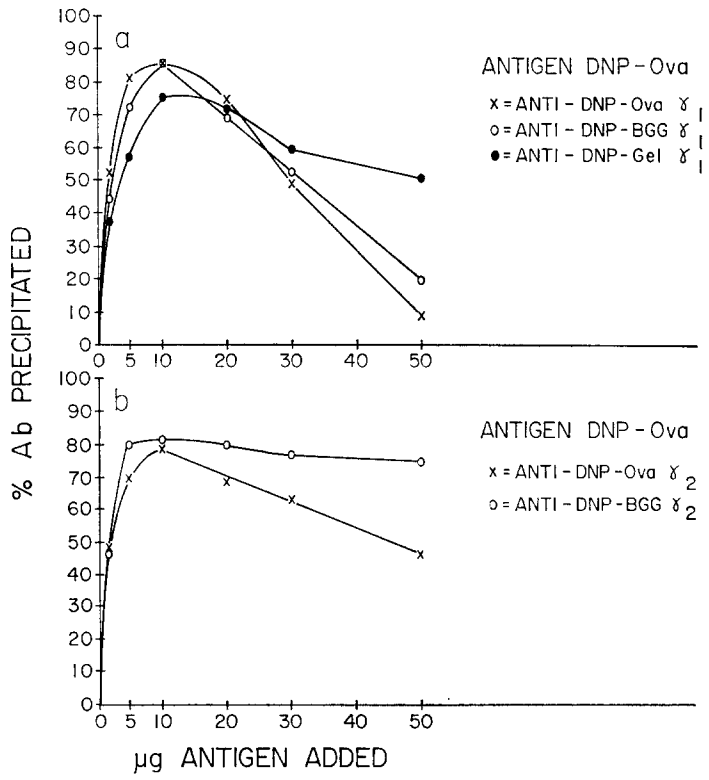

FIG. 4. Precipitin curves of purified guinea pig γ_1 anti-DNP-Ova, anti-DNP-BGG, and anti-DNP-Gel with DNP-Ova (4 *a*), and γ_2 anti-DNP-Ova, and anti-DNP-BGG with DNP-Ova (4 *b*). Precipitin reactions were carried out in a total volume of 0.2 ml with an initial antibody (Ab) concentration of 400 μg/ml in PBS. Reactions mixture were incubated at 37°C for 1 hr and then at 4°C for 48 hr.

used throughout these studies were purified by precipitation with DNP-BF and subsequent elution with dinitrophenol. Thus, no antibodies directed solely at carrier protein exist in these preparations. Fig. 4 *b* shows a similar comparison of γ_2-anti-DNP-Ova and γ_2-anti-DNP-BGG antibodies precipitated again with DNP-Ova with comparable results. In this case, as would be expected from the charge properties of the antibody (1, 17), inhibition of precipitation in antigen excess was less striking. Nevertheless, the precipitation of the anti-

DNP-Ova antibody in antigen excess was again inhibited more than was that of anti–DNP-BGG.

DISCUSSION

Previous attempts to study the immunological specificity of antihapten antibodies for areas of the carrier molecule adjacent to the site of attachment of the hapten have generally relied on precipitation techniques and have shown that, in adequately chosen systems, more antibody could be precipitated by the immunizing antigen than by conjugates of the same hapten with different proteins (8–10). This is true even when the immunizing antigen was a conjugate of a nonimmunogenic protein such as homologous serum albumin (10). Additional evidence for specificity of antihapten antibodies for the carrier molecule is provided by the precipitin curves presented in this study. The precipitation of specifically purified anti-DNP γ_1- or γ_2-antibodies by DNP-Ova was shown to be inhibited by excess antigen to a significantly greater degree in the case of anti–DNP-Ova than in the case of anti–DNP-BGG antibodies in spite of similar affinities for ϵ-DNP-L-lysine. To interpret these results, one must consider that inhibition of precipitation is caused by the formation of smaller complexes with a higher ratio of antigen to antibody than is present in precipitating aggregates of the same components. The greater tendency to form soluble complexes observed with anti–DNP-Ova than with anti–DNP-BGG when the same excess of DNP-Ova is added can be interpreted as reflecting a more efficient binding of DNP-Ova by anti–DNP-Ova as compared with anti–DNP-BGG. Furthermore, because of differences in their microenvironments all of the DNP groups present in a DNP-protein should not be regarded as identical. It is most probable that certain groups predominate as antigenic determinants. Therefore, in the immunizing antigen a small number of DNP groups are present which served as the actual antigenic determinants and so interact more strongly (preferentially) with the homologous antibody than do the remaining DNP groups. It is probable that when DNP antibody is reacted with a DNP conjugate other than the immunizing antigen no such exceptionally reactive DNP groups are present. This difference would favor formation of small soluble aggregates in antigen excess when the immunizing antigen, as compared with another DNP-conjugate, is used in the precipitin reaction.

Besides definite evidence for "carrier" specificity of purified antihapten antibodies, the data presented also provide measurements of the energy contribution which can be attributed to carrier specificity in the system studied. These values were obtained from fluorescence-quenching titrations of anti–DNP-PLL antibodies and of anti–DNP-protein antibodies with $DNP_{0.6}$-PLL_{240} and with ϵ-DNP-L-lysine. In spite of the demonstrated similarity of $DNP_{0.6}$-PLL_{240} with the more highly conjugated DNP-PLL used for immunization, some differences in the microenvironments of DNP groups probably

exist between the two molecules. Thus, the $DNP_{0.6}$-PLL_{240} used in fluorescence-quenching titrations must be considered as a highly cross-reactive, and not strictly homologous, antigen. The measurements reported here of the energy contributed by carrier specific interactions should therefore be viewed as a minimum estimate of energy of carrier specificity for this system.

The data presented show that intact antibodies of anti–DNP-PLL specificity have energies of interaction with $DNP_{0.6}$-PLL_{240} of 0.8 to 2.1 kcal/mole greater than with ϵ-DNP-L-lysine whereas intact antibodies of anti–DNP-protein specificity have energies of interaction with $DNP_{0.6}$-PLL_{240} of up to 2.4 kcal/mole less than with ϵ-DNP-L-lysine. The data also indicate that carrier specificity is not confined to antibodies of DNP-PLL specificity. The decrease in energy of binding of $DNP_{0.6}$-PLL_{240} as compared to energy of binding of ϵ-DNP-L-lysine varied with antibodies produced using different protein carriers. $\Delta(\Delta F^0)$ was significantly more positive for anti–DNP-BSA antibodies than for anti–DNP-BGG or anti–DNP-Ova antibodies. Indeed, two anti–DNP-Gel antibodies exhibited small negative values for $\Delta(\Delta F^0)$ indicating a degree of structural complementariness between these anti–DNP-Gel antibodies and parts of the PLL molecule. This may well be related to the lack of rigidity in the structure of gelatin which might cause it to resemble PLL fairly closely.

$DNP_{0.77}$–PDL_{109} caused quenching of fluorescence of anti–DNP-PLL antibodies intermediate between that observed with $DNP_{0.6}$-PLL_{240} and with ϵ-DNP-L-lysine demonstrating a degree of structural similarity between the homopolymers of optical isomers of lysine (Fig. 1).

In the course of these experiments some information regarding the nature of the repulsive forces between anti–DNP-BGG antibodies and $DNP_{0.6}$-PLL_{240} has been obtained. Increasing the salt concentration nearly abolished the positive $\Delta(\Delta F^0)$ for intact anti–DNP-BGG. However, similar measurements made using Fab′ fragments (so as to exclude the effect of cooperation which will be discussed) showed that $\Delta(\Delta F^0)$ was changed from +1.96 kcal/mole to +1.04 kcal/mole. Thus electrostatic forces appear to be largely but not exclusively involved in the diminished binding of $DNP_{0.6}$-PLL_{240} by anti–DNP-BGG antibodies. An additional factor which might contribute to the decreased binding of $DNP_{0.6}$-PLL_{240} by anti–DNP protein antibodies has been suggested previously by Levine (25). As a result of the lack of conformational complementarity of the heterologous carrier for the antibody-binding site the specific binding of the hapten itself is interfered with on the basis of what is essentially steric inhibition. Interaction between hapten and carrier, as suggested by Singer (18) may also explain some of the effects observed.

Titrations with $DNP_{0.6}$-PLL_{240} which contains numerous PLL molecules with more than one DNP group per molecule allowed the demonstration of a cooperative effect between the two antigen-binding sites of divalent antibody. This cooperative effect was observed and its magnitude estimated by comparing equilibrium measurements made with intact antibody and with univalent

Fab′ fragments. Fab′ fragments of anti–DNP-PLL antibodies bound $DNP_{0.6}$-PLL_{240} with about 1 kcal/mole less energy than did intact anti–DNP-PLL. Similarly, Fab′ fragments of anti–DNP-BGG specificity had a ΔF^0 with $DNP_{0.6}$-PLL_{240} of 0.6 kcal/mole less energy than exhibited by intact anti–DNP-BGG antibody. This cooperative effect does not appear to result from a conformational change in the antibody molecule incident to binding of one site (allosteric changes) which results in an increased affinity of the second site since no difference in the ΔF^0's for interaction with the univalent hapten, ϵ-DNP-L-lysine, was demonstrated. From data available at present it would appear most likely that the cooperative effect is due to facilitation of binding of the second site of a bivalent antibody molecule to the same multivalent antigen molecule to which the first site has already bound. As a result of the binding of one antigenic determinant to one antibody site on a divalent antibody the bulk of those forces opposing the apposition of the two macromolecules have been overcome. These forces probably include such factors as electrostatic repulsion, entropy effects, and steric hindrance. The extent of this cooperative effect in the system studied is of the order of 0.6 to 1 kcal/mole of sites and twice as much for divalent antibody molecules.

Cooperative effects of this type may have considerable biologic importance. Many naturally occurring substances that elicit immune responses have repeating antigenic units (viruses, cell surfaces, and proteins consisting of multiple polypeptide chains). Thus an opportunity for both valences of antibody to combine with a single antigen particle exists in many biologically important systems. In fact, binding of both combining sites of an IgG antibody molecule to a single particle of antigen has been reported for viruses (19), erythrocytes (20), and ferritin (21). The efficiency of binding would be significantly increased by the additional energy of interaction resulting from the cooperative effects described above. In IgM antibodies, which appear to have 5 valences (22–24) cooperative effects can be predicted to be of even greater magnitude.

The $\Delta(\Delta F^0)$ measured with an Fab′ fragment of anti–DNP-PLL specificity was -0.41 kcal/mole. In the strictest sense, this is the energy of interaction contributed directly by the carrier. However, when account is taken of the decrease in binding energy exhibited by anti-DNP antibodies formed against other DNP conjugates and of the cooperative effects present in intact divalent antibody, carrier specificity may be viewed as contributing energies of the order of 2 to 3 kcal/mole which represent approximately 30% of the total energy of binding. This energy increment may indeed be sufficient to explain many of the observed requirements for "carrier" specificity in biological reactions.

SUMMARY

Equilibrium measurements of interactions of anti-DNP antibodies, prepared using DNP-PLL and several DNP-proteins for immunization, with $DNP_{0.6}$-

PLL_{240} and with the univalent hapten, ε-DNP-L-lysine, were made utilizing the technique of fluorescence quenching. Carrier specificity of anti–DNP-PLL antibodies was demonstrated by a higher average intrinsic association constant (K_0) of anti–DNP-PLL antibodies with $DNP_{0.6}$-PLL_{240} than with ε-DNP-L-lysine. The free energy contribution of the PLL carrier to the interaction of intact anti–DNP-PLL antibodies with $DNP_{0.6}$-PLL_{240} was from −0.8 to −2.1 kcal/mole. On the other hand, intact anti–DNP-protein antibodies displayed a lower energy of interaction with $DNP_{0.6}$-PLL_{240} than with ε-DNP-L-lysine of up to +2.4 kcal/mole.

Fab′ fragments of both anti–DNP-PLL and anti–DNP-BGG antibodies have K_0's with ε-DNP-L-lysine identical to the K_0's of the intact anti–DNP antibodies from which they were prepared. However, K_0 of interaction of Fab′ fragments with $DNP_{0.6}$-PLL_{240} (a large proportion of the conjugated PLL molecules in this preparation bear more than one DNP group) is considerably lower than that of the intact antibody. Thus a cooperative effect in the binding of bivalent antibody and bivalent (or greater) antigen exists and is of the order of −1.2 to −2.0 kcal/mole of IgG antibody.

Although the direct contribution of the carrier to the interaction of Fab′ fragment of anti–DNP-PLL and $DNP_{0.6}$-PLL_{240} is −0.4 kcal/mole, the energy of carrier specificity, based upon consideration of cooperative effects and of repulsion of anti-DNP-protein antibodies for portions of the DNP-PLL determinants, is of the order of −3 kcal/mole (approximately 30% of total binding energy).

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