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

I. Effect of Carrier on the Nature of the Antibody Synthesized

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The carrier to which a hapten is coupled can influence both the nature and the immunological specificity of the immune response to the hapten. Thus, guinea pigs genetically capable of an immune response to haptens attached to the L-lysine homopolymer (1, 2) fail to respond to the same haptens coupled to poly-D-lysine (3, 4). Evidence for definite "carrier specificity," that is, immunologically specific interactions involving portions of the carrier molecule adjacent to the attached hapten, has been clearly demonstrated with regard to the ability of hapten-protein conjugates to stimulate a secondary response in rabbits (5) or to elicit delayed hypersensitivity reactions in sensitized guinea pigs (6, 7). In addition, various degrees of carrier specificity have also been observed for several antigen-antibody reactions: wheal and flair reactions in man (8), passive cutaneous anaphylaxis in guinea pigs with early immunization sera (9), some Arthus reactions (10), and precipitin reactions in liquid media in selected systems (11–13).

The present studies were undertaken to define, in quantitative terms, the effect of the carrier upon the antibody response to the 2,4-dinitrophenyl (DNP) determinant. In this paper, the effect of varying the carrier molecule upon the immunoglobulin class, the amount, and the average intrinsic association constant (K_0) of the antibody produced, has been investigated in the guinea pig. In a second paper (14), the specificity of guinea pig anti–2,4-dinitrophenyl-poly-L-lysine antibodies for the poly-L-lysine carrier is demonstrated and the amount of energy contributed to the antibody-antigen interaction by specific binding of the carrier molecule is estimated.

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Materials and Methods

Proteins and Polypeptides.—Bovine gamma globulin (BGG), bovine serum albumin (BSA), and bovine fibrinogen (BF) were obtained from Armour Pharmaceutical Co., Kankakee, Illinois. Hen ovalbumin (Ova), 2 × recrystallized was obtained from Pentex Corp., and calf skin gelatin (Gel) (pK_I = 4.9) from Eastman Organic Chemicals, Rochester, New York. Guinea pig serum albumin (GPA) was prepared by starch block electrophoresis as previously described (15, 16). Purity was established by immunoelectrophoresis. Poly-L-lysine (PLL) obtained from Pilot Chemical Company, Watertown, Mass., had an average of 240 residues per molecule. PLL with 1420 residues per molecule was obtained from Mann Research Laboratories, New York.

Other Reagents.—*N*,ε-2,4-dinitrophenyl-L-lysine (ε-DNP-L-lysine) was purchased from Cyclo Chemical Corporation, Los Angeles. 2,4-dinitrophenol (DNP-OH) was obtained from Fisher Scientific Corporation, New York, and recrystallized from water before use. ε-Aminocaproic acid (EACA) was obtained from Mann Laboratories. Sodium lauryl sulfate was recrystallized from 95% ethanol before use. 2,4-dinitrofluorobenzene (DNFB) was obtained from Eastman Organic Chemicals.

Preparation of Dinitrophenylated Proteins.—Dinitrophenylated (DNP) proteins and polylysines were prepared essentially as described previously (12, 17, 18) by reaction of 2,4-dinitrofluorobenzene with protein under alkaline conditions. Conjugated proteins were purified by extensive dialysis. Protein concentrations were determined by Kjeldahl analysis and the approximate degree of substitution was calculated from the absorbancy of the derivative at 360 mμ by use of the known absorbancy of free ε-DNP-L-lysine ($\epsilon_{1\,cm}$, at 360 mμ ≈ 17,400, see reference 19).

The following DNP-derivatives were prepared: DNP_{62}-BGG, DNP_{70}-BGG, DNP_{169}-BF, 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}. Subscripts refer to average number of groups per molecule of carrier[1] except in the case of gelatin, a nonhomogeneous protein, where average numbers of groups per assumed 100,000 molecular weight is reported.

Immunization of Guinea Pigs.—Hartley strain guinea pigs were immunized by injection of 1 mg of DNP protein. The antigen solutions were emulsified with equal volumes of complete Freund's adjuvant (20) (Difco preparation containing heat-killed *Mycobacterium butyricum*, Difco Laboratories, Inc., Detroit) and injected into the four foot-pads (total volume 0.4 ml). Animals were boosted, by four intradermal injections of 100 μg of antigen in saline, four times at weekly intervals, starting 9 days after the initial injection. Animals were bled 6 days after the final injection.

Immunization with DNP_{11}-PLL_{240}, DNP_{32}-PLL_{1420}, and DNP_{44}-PLL_{1420} was carried out with an initial injection of 100 μg of antigen in complete Freund's adjuvant with subsequent booster injections of 10 to 100 μg of antigen according to a schedule similar to that described above for the DNP protein antigens.

Precipitin Curves.—Precipitin curves were carried out according to the method of Eisen et al. (12, 21). Reaction mixtures were incubated at 37°C for 1 hr and then held at 4°C for 2 days before analysis. Washed specific precipitates were dissolved in 0.02 M sodium lauryl sulfate and absorbancies at 278 and 360 mμ were determined. The amount of antibody precipitated was calculated from the 278 mμ absorbancy, after correcting for the antigen present as indicated by the 360 mμ absorbancy. The extinction coefficient ($E^{1\%}_{1\,cm}$) for guinea pig anti-DNP antibody at 278 mμ was taken as 13.2 (22).

When precipitin curves were carried out using purified antibody the 360 and 278 mμ

[1] For calculation of the degree of substitution molecular weights of proteins were taken as follows: BGG, 150,000; BF, 330,000; BSA, 69,000; Ova, 44,000; and GPA, 69,000.

absorbancies of the supernate remaining after removal of specific precipitates by centrifugation were determined and used to calculate the per cent precipitation of the antibody initially present.

Purification of Antibody.—Antibody specific for the DNP-determinant was purified by extraction of washed specific precipitates, formed at equivalence with DNP-BF (in the presence of 0.01 M disodium ethylenedinitrilo tetraacetate) with 0.1 M DNP-OH according to the methods of Farah et al. (23). The antigen was removed by streptomycin precipitation (35 mg streptomycin/ml) and the DNP-OH was removed by dialysis and passage through a Dowex 1-× 8 (200 to 400 mesh) anion exchange column.

The purified antibody preparations showed minimal contamination with hapten; they were essentially colorless and had absorbancies at 360 mμ of less than 3% that at 278 mμ. They were 85 to 90% precipitable with DNP-BGG at an initial antibody concentration of 400 μg/ml. On immunoelectrophoresis they exhibited only γ_1- and γ_2-lines when developed with rabbit anti-guinea pig whole serum. Yields of purified antibody were generally 50 to 60% of that present in the serum.

Separation of γ_1- and γ_2-Guinea Pig Anti-DNP Antibodies.—Anti-DNP antibodies were separated into γ_1- and γ_2-components by ion exchange chromatography using DEAE-cellulose (22). Purified antibody preparations were dialyzed against 0.01 M potassium phosphate buffer, pH 8.0. A small amount of euglobulin antibody precipitated during dialysis and was removed by centrifugation prior to chromatography. Approximately 10 mg of antibody were applied to a 1 × 10 cm column of DEAE-cellulose equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, and the γ_2-antibody was eluted with the same buffer. When no further 278 mμ absorbing material could be eluted from the column with the 0.01 M buffer, 0.15 M potassium phosphate buffer, pH 8.0, was passed through the column and the γ_1-antibody eluted.

The antibody fractions were all examined by immunoelectrophoresis and were found to contain, within the limits of sensitivity of the method, exclusively γ_1- or γ_2- globulins.

Preparation of ^{3}H-DNP-ε-Aminocaproic Acid (^{3}H-DNP-EACA).—Tritiated 2,4-dinitrochlorobenzene was obtained from New England Nuclear Corp., Boston, and used to prepare ^{3}H-DNP-EACA by reaction with ε-aminocaproic acid under alkaline conditions. The product was purified by repeated acid precipitation and finally crystallized from a slightly acidified aqueous solution. Specific activity of the preparation when counted in Bray's solution (24) using a Packard Tricarb liquid scintillation counter was 13.9 mc/mM.

Measurement of Antibody-Binding Affinity by Equilibrium Dialysis.—Equilibrium dialysis was performed as described by previous workers (25). 1 ml of antibody solution in phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.6) was dialyzed against 1 ml of ^{3}H-DNP-EACA. Antibody concentrations between 100 and 400 μg/ml were used. Tubes were rotated at 31°C for 18 hr after which samples were removed from inside and outside the dialysis sac and counted in a Packard Tricarb liquid scintillation counter as described above.

K_0 was calculated from equilibrium dialysis data according to the Sips equation (26, 27): $\text{Log } r/n - r = a \log c + a \log K_0$ where r is fraction of antibody sites bound, n is valence (2 for γ_1- and γ_2-guinea pig antibody), c is free hapten concentration, and a is Sips heterogeneity index.

Measurement of Antibody-Binding Affiinity by Fluorescence Quenching.—Affinities of purified anti-DNP antibodies for ε-DNP-L-lysine were calculated from the quenching of antibody fluorescence upon binding of hapten (28). The technique of titration and method of calculation of average association constants from the data obtained have been described in detail by Eisen and Siskind (29) for rabbit anti-DNP antibody. In the present work, titrations were carried out on 2 ml samples of antibody solution at a concentration of 30 μg/ml in PBS. The fluorescence of antibodies at 360 mμ when excited at 280 mμ was measured in a thermostated Aminco-Bowman spectrophotofluorometer. All determinations, unless otherwise indicated,

were carried out at 26°C. In the course of calculations the following values were used: (*a*) $E^{1\%}_{1\,cm}$ at 278 mμ for guinea pig γ_2-antibody in neutral solution was taken as 13.2, for guinea pig γ_1-antibody as 15.0, and for unfractionated antibody (predominantly γ_2) as 13.2 (22); and (*b*) the molecular weight of guinea pig antibody was taken as 150,000. Results recorded represent averages of duplicate determinations.

Calculations were carried out using a Control Data Computer. The computer program was originally written by Mrs. Elinor Murphy and adopted for use with the Control Data system of the Department of Neurology, Section on Communication Sciences, New York University Medical Center by Mr. Robert Bloom.

In order to apply this method, the degree of quenching of antibody fluorescence when all antibody combining sites are occupied by hapten (Q_{max}) must be determined for the specific antibody-hapten system under investigation. In the present studies this was determined, as

TABLE I

Effect of Different Assumed Values for Maximum Possible Quenching (Q_{max}) upon the Equilibrium Constant Calculated

Assumed Q_{max}	$K_0 \times 10^{-6}$*
%	
75	5.8
80	3.5
85	2.6
90	1.9
95	1.5
100	1.2
From equilibrium dialysis data	0.67

* $K_0 \times 10^{-6}$ in liters/mole in PBS at 31°C for binding of ^{3}H-2,4-dinitrophenyl-ε-aminocaproic acid by a pool of purified anti-DNP antibody from 4 animals immunized with DNP-BGG.

in previous work (29), by comparing the association constants calculated from fluorometric titration data, using different assumed values for maximum quenching, with equilibrium constants determined independently by equilibrium dialysis. The results of calculations based upon different assumed values for Q_{max} are tabulated in Table I. By equilibrium dialysis (using the same hapten preparation as used for the fluorescence quenching) the association constant (K_0) of this antibody preparation for DNP-EACA was found to be 6.7×10^5 liters/mole. Thus, the best agreement with the results of equilibrium dialysis is obtained when one assumes a Q_{max} of 100%. Such comparisons were carried out with four different antibody preparations: (*a*) anti-DNP-BSA, (*b*) anti-DNP-BGG, (*c*) γ_1-fraction of anti-DNP-BSA, and (*d*) γ_2-fraction of anti-DNP-BGG. In every case the best agreement with equilibrium dialysis was obtained using Q_{max} of 100%. A comparison of the results obtained with equilibrium dialysis and with fluorescence quenching (Q_{max} taken as 100%) is shown in Table II and in Fig. 1. In each case it can be seen that the value of K_0 obtained by fluorescence quenching is 2 to 3 times greater than that calculated from equilibrium dialysis. (It should be noted that standing at 31°C for 18 hrs did not affect the K_0 obtained by fluorescence quenching. Thus, the time difference between the two methods could not account for the differences observed in K_0.) This 2-fold factor does not affect the validity of comparing different antibody preparations and

does not affect calculations of energy differences between different antibody preparations. Therefore, the data reported in this and in the accompanying paper (14) are calculated assuming a Q_{max} of 100% and values for the molecular weight and extinction coefficients given above. The values reported should be understood to be about 2-fold higher than what would be obtained by equilibrium dialysis. Although we cannot critically account for the small but consistent discrepancy between the results of fluorescence quenching and equilibrium dialysis several possible factors may be mentioned: (*a*) If the quantum yield of energy transfer differed somewhat with antibodies of different affinity, the known heterogeneity of antibody affinity (27) might contribute to the discrepancy observed. (*b*) The quenching of more than half of the fluorescence of each bivalent antibody molecule by the first hapten molecule bound would also tend to give somewhat high values for K_0 by fluorescence quenching. This is the most probable explanation since the degree of quenching produced by the first few additions of ligand often gives the impression that more sites are bound than ligand molecules added (29, 30). This is, of course, impossible and this appearance is probably due to the fact that the first molecule of hapten bound quenches more than half of the antibody fluorescence.

TABLE II

Binding of ^{3}H-DNP-ε-Aminocaproic Acid by Guinea Pig Antibody Determined by Equilibrium Dialysis and Fluorescence Quenching

Preparation*	$K_0 \times 10^{-5}$‡	
	Fluorescence quenching§	Equilibrium dialysis
Anti-DNP-BGG	12.	6.7
Anti-DNP-BSA	3.2	1.5
Anti-DNP-BGG, γ_2	9.6	3.0
Anti-DNP-BSA, γ_1	8.4	3.3

* Pools of purified antibody from groups of 4 guinea pigs were used.

‡ $K_0 \times 10^{-5}$ in liters/mole determined in PBS at 31°C.

§ Calculated using $Q_{max} = 100\%$.

Despite the theoretical difficulties discussed above the differences between the values obtained by the two methods of measurement are small and consistent and the fluorescence-quenching technique has been empirically justified both in the present paper and by previous workers (28, 29). It can be regarded as capable of giving accurate association constants for the antibody-hapten system studied.

RESULTS

Effect of Carrier on the Amount of Anti-DNP Antibody Synthesized.—Groups of guinea pigs were immunized with 4 different dinitrophenylated proteins: DNP-BGG, DNP-BSA, DNP-Ova, and DNP-Gel. The concentration of antibodies having DNP specificity present in the sera of individual guinea pigs was determined by quantitative precipitin tests using DNP-BF as precipitating antigen. DNP-BF was chosen for this purpose because it is relatively insoluble and so brings about maximum possible precipitation of specific anti-DNP antibody present and because the fibrinogen carrier does not significantly

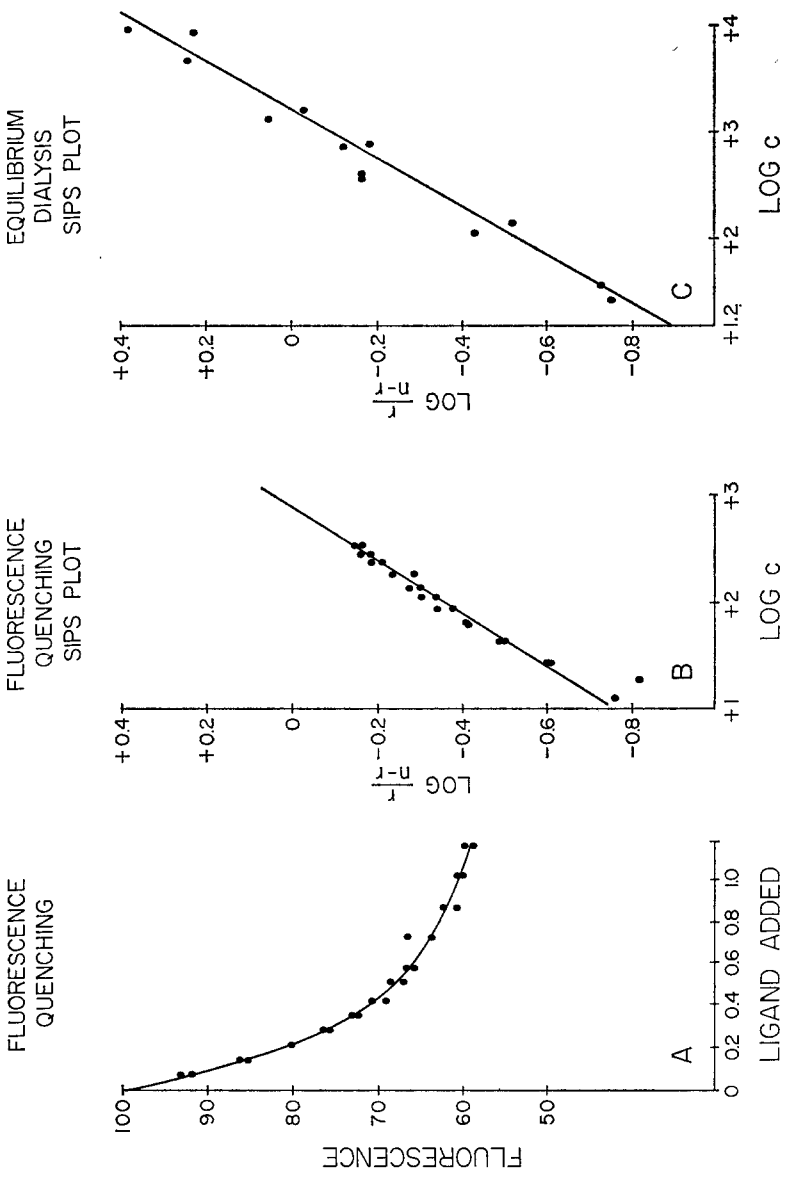

FIG. 1. Binding of ^{3}H-2,4-dinitrophenyl-ε-aminocaproic acid by purified guinea pig anti-DNP antibody at 31°C in PBS.
A. Fluorescence quenching using 2 ml of antibody at a concentration of 30 μg/ml.
B. Sips plot (28, 29) of data from fluorescence quenching experiment illustrated in A.
C. Equilibrium dialysis results plotted according to the Sips method using the same antibody preparation and hapten.

cross-react with antibody to any of the carriers used for immunization. The concentration of anti-DNP antibodies in the serum of individual guinea pigs immunized with different dinitrophenylated carriers is tabulated in Table III. It is immediately apparent that the amount of antibody produced varies greatly depending on the carrier used for immunization. DNP-BGG elicited

TABLE III

Effect of Carrier on Amount and Species of Anti-DNP Antibody Produced by Individual Guinea Pigs

Immunizing antigen							
DNP-BGG		DNP-BSA		DNP-Ova		DNP-Gel	
mg Ab/ml*	$\%\gamma_2$‡	mg Ab/ml	$\%\gamma_2$	mg Ab/ml	$\%\gamma_2$	mg Ab/ml	$\%\gamma_2$
3.79	88	3.63	19	3.50	58	2.75	51
5.50	73	3.50	37	4.41	37	2.05	17
4.74	95	1.52	25	3.82	45	1.94	30
4.51	43	1.18	37	2.21	—	0.12	—
4.18	91	1.63	—			1.17	—
6.36	73	2.38	—				
6.48	—	1.32	—				
3.81	—	0.98	—				
5.18	—	2.12	—				
6.78	—	0.17	—				
6.40	—	1.97	—				
2.08	—	3.09	—				
5.06	—	1.10	—				
—	76						
—	76§						
—	92						
Mean 4.99	79	1.89	30	3.49	47	1.61	33

— Determination not performed.

* Determined by quantitative precipitin reaction using DNP-BF as antigen.

‡ Determined from yields of γ_1- and γ_2-antibodies after fractionation by ion exchange chromatography.

§ Pool of antibody purified from sera of 4 guinea pigs.

an average of 5 mg anti-DNP antibody/ml as compared with 3.5 mg/ml elicited by DNP-Ova and less than 2 mg/ml when BSA or Gel were used as carriers.

Effect of Carrier on the Immunoglobulin Class of Antibody Formed.—It is well known that two different species of guinea pig 7S antibodies exist which can be separated electrophoretically (16). They are referred to as γ_1 and γ_2, the γ_1 being the more highly charged at neutral pH. From examination of immunoelectrophoretic patterns of the purified antibody preparations, it was qualita-

tively apparent that the relative amounts of γ_1- and γ_2-antibodies synthesized differed with different carriers. Purified antibodies from individual animals were therefore separated into γ_1- and γ_2-components by ion-exchange chromatography. The approximate per cent of the antibody of γ_2-class is listed in Table III for individual animals. Depending on the carrier used for immunization the per cent γ_2 was found to vary from 80% with BGG as carrier to 30% with BSA as carrier.

TABLE IV

Effect of Carrier on Affinity ($K_0 \times 10^{-5}$) of Anti-DNP Antibody for ε-DNP-L-Lysine*

Immunizing Antigen					
DNP-BGG	DNP-BSA	DNP-PLL	DNP-Ova	DNP-Gel	DNP-GPA
270.	1.3	5.0	1.8	4.7	1.5
27.	3.9	17.	5.5	40.	0.81
31.	4.7	47.	2.5	7.6	2.7
11.	1.1	40.	5.5	8.1	2.5
140.	4.9	30.	34.	3.5	0.43
37.	11.	61.	3.0	7.7	1.9
15.	3.9	4.9	3.9	4.5	5.0
24.	5.8	32.	67.	4.5	
31.	3.9	11.	82.		
55.	8.0		7.0		
28.	6.3		27.		
20.	5.5				
23.	3.5				
57.					
Mean 55.	4.9	27.	22.	10.	2.1
Median 30.	4.7	30.	5.5	6.2	1.9

* $K_0 \times 10^{-5}$ in liters/mole determined by fluorescence quenching in PBS at 26°C. Each determination recorded was carried out on the purified antibody of individual guinea pigs. Values recorded are averages of duplicate determinations.

Effect of Carrier on the Association Constant of the Antibody Formed.—Antibody was specifically purified from the sera of guinea pigs immunized with DNP on different carriers. The association constant for the binding of the homologous ligand, ε-DNP-L-lysine[2], was determined from the quenching of antibody fluorescence. The results are presented in Table IV. Determinations were all carried out on antibody purified from sera of individual guinea pigs. It is clear that the affinity of the antibody produced is greatly affected by the

[2] ε-DNP-L-lysine is known to correspond to the major antigenic determinant present in dinitrophenylated proteins. At least 95% of the DNP groups in such proteins have been shown to be coupled to the ε-amino groups of lysine residues (17).

carrier used in immunization. A 10-fold difference in K_0 was found between the anti-DNP antibody formed when BGG was used as carrier as compared with that formed when BSA or GPA were used. Antibodies induced with DNP

TABLE V

Association Constants for Binding of ε-DNP-L-Lysine by γ_1- and γ_2-Antibodies Obtained from Sera of Individual Guinea Pigs

Immunizing antigen	$K_0 \times 10^{-5}$*	
	γ_1	γ_2
DNP-BGG	>1000. ‡	340.
	14.	35.
	8.9	22
	41.	17.
	19.	28.
	9.5	31.
Average	19.	27.
DNP-BSA	0.62	1.6
	10.	2.9
	10.	5.2
	7.2	1.3
	2.9	3.5
Average	6.1	2.9
DNP-Ova	45.	37.
	62.	3.2
	30.	8.9
Average	46.	16.
DNP-Gel	3.8	4.0
	11.	1.9
	4.2	3.5
Average	6.3	3.1

* $K_0 \times 10^{-5}$ in liters/mole in PBS at 26°C. Each pair of values represents antibody purified and fractionated from the sera of an individual guinea pig.

‡ This value was not included in calculating the average values listed as determinations in this range are highly inaccurate.

derivatives of PLL, Ova, and Gel were intermediate in affinity. By *t* test (31), the anti-DNP-BGG antibody was, statistically, of significantly greater affinity than the antibody formed in response to DNP-BSA or to DNP-GPA ($P < 0.05$). Furthermore, anti-DNP-PLL antibodies had affinities significantly

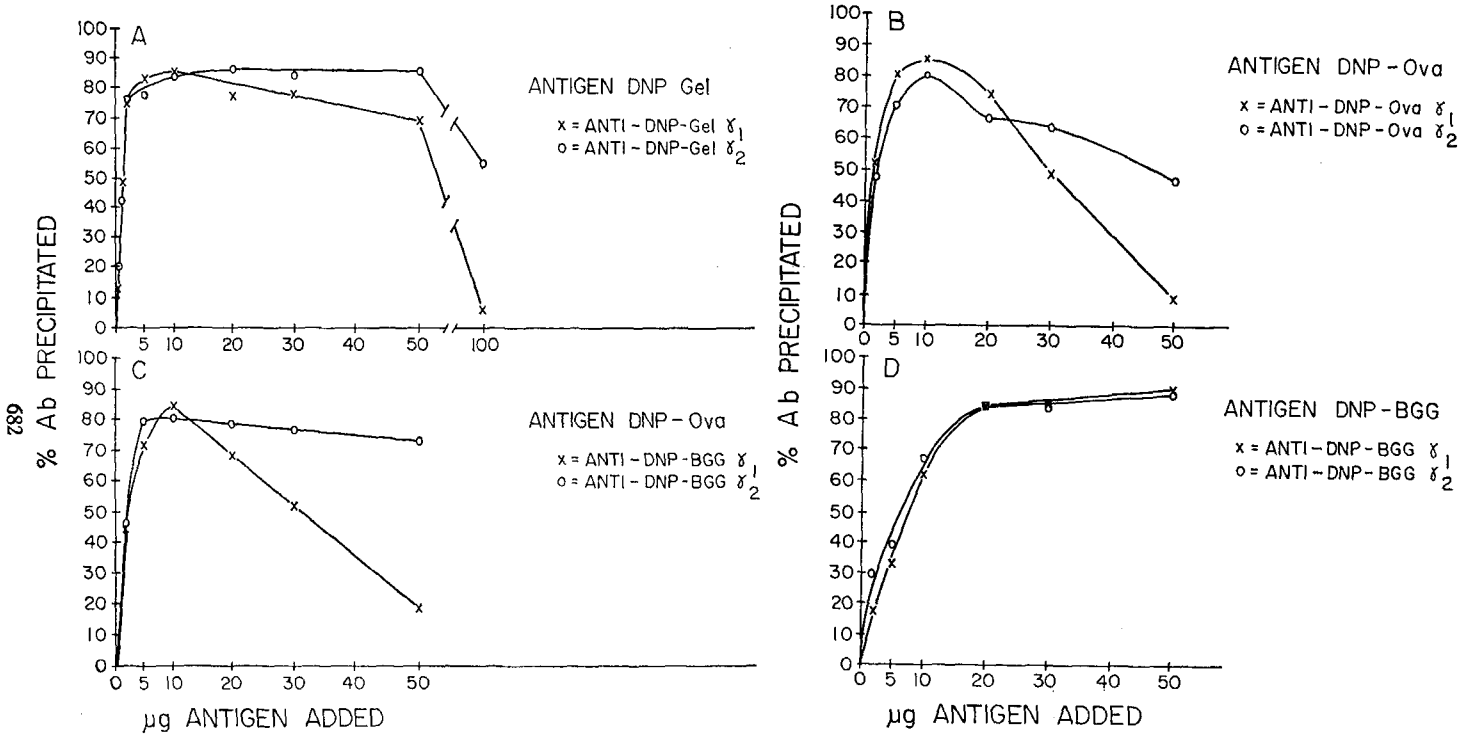

FIG. 2. Precipitin curves for purified guinea pig γ_1- and γ_2- anti-DNP antibodies with different DNP proteins. Results expressed as per cent of total antibody precipitated as determined by spectral analysis of supernates after removal of precipitates by centrifugation. Initial antibody concentration was 400 μg/ml and total volume of the reaction mixture was 0.2 ml.

higher than anti–DNP-Gel ($P < 0.05$), anti–DNP-BSA ($P < 0.01$), and anti–DNP-GPA ($P < 0.01$). Anti–DNP-Ova antibodies had affinities significantly higher than anti–DNP-BSA ($P < 0.05$).

Association Constants of the γ_1- and γ_2-Antibody Fractions.—Since it was shown (Table III) that the relative amounts of γ_1- and γ_2-antibodies differed when different carriers were used, the possibility existed that the two antibody classes were of different affinity and the differences in K_0 resulting from use of different carriers were merely due to the presence of γ_1- and γ_2-antibodies in different proportions. Binding constants of the fractionated antibodies from individual guinea pigs were therefore determined and are shown in Table V. The fractionation procedure did not affect binding strength as the original antibody, in every case, had a K_0 very similar to that of the γ_1- and γ_2-fractions, generally intermediate between them. It is apparent that there is relatively little difference between the affinities of γ_1- and γ_2-antibodies from the same guinea pig. With anti–DNP-BGG antibodies both γ_1- and γ_2-components had high affinity while with anti–DNP-BSA antibodies both fractions had low affinity. In one third (6 out of 17) of the pairs studied the two fractions can be regarded as identical in affinity within the limits of sensitivity of the technique. One can generally regard a difference in K_0 of more than 2-fold as significant. By this criterion, in 5 out of 17 pairs measured, the γ_1-antibody was of slightly greater affinity (2- to 5-fold). In 4 out of 17 pairs the γ_2 was of slightly greater affinity than the γ_1. In only two cases the γ_1-antibody was of considerably higher affinity than the γ_2-fraction.

Precipitin Curves with γ_1- and γ_2-Antibodies.—Precipitin curves for purified γ_1- and γ_2-antibody fractions with several different DNP-proteins are illustrated in Fig. 2. The curves obtained with γ_1- and γ_2-antibodies correspond closely up to the equivalence point. In antigen excess, the γ_1-antibody exhibits far greater inhibition of precipitation than does the corresponding γ_2-antibody. With DNP-BGG, a relatively insoluble protein, as precipitating antigen, no inhibition was observed in antigen excess with either antibody type.

DISCUSSION

In the course of the present work the method of fluorescence quenching (28, 29) has been applied to the measurement of affinity of guinea pig antibodies specific for the ϵ-2,4-dinitrophenyl-L-lysyl determinant. The method was calibrated by comparison with equilibrium dialysis. It was found that an assumption of 100% for the maximum possible quenching of guinea pig antibody when all sites were occupied by hapten gave the best agreement with the reresults of equilibrium dialysis. It has been previously reported for the rabbit anti-DNP system that a maximum quenching of 72.5% gives the best agreement with equilibrium dialysis (29). The difference between the efficiency of energy transfer (quenching) for rabbit and guinea pig anti-DNP antibody

emphasizes the importance of empirically determining Q_{max} whenever this method is to be applied to a new antibody-hapten system even if the only difference is the species from which the antibody is derived. It was found that guinea pig anti-DNP antibody, regardless of what carrier was used for induction of antibody synthesis and regardless whether it was of γ_1- or γ_2-mobility, had the same Q_{max}. Whether or not these convenient similarities in Q_{max} will remain when tested for other guinea pig antibody-hapten systems cannot be answered at present.

In this paper we have systematically examined the effect of the carrier on antibody production. It is very clear, from the data presented, that the amount of antihapten antibody formed and the relative amounts of γ_1- and γ_2-antibodies formed are markedly influenced by the carrier used for immunization. It is furthermore clear that the affinity of the antibody for the homologous haptenic determinant, ϵ-DNP-L-lysine, is greatly influenced by the carrier molecule. This effect of the carrier on the affinity of the antibody synthesized has been shown to be unrelated to the relative amounts of γ_1- and γ_2-antibodies present since these two antibody fractions are generally very similar in binding constant. Animals immunized with DNP-BGG produced both γ_1- and γ_2-antibodies of high affinity while animals immunized with DNP-BSA produced antibody of relatively low affinity of both classes. The similarity of affinities in γ_1- and γ_2-antibodies produced by the same animal is reasonable in light of what is known about antibody structure since the difference between γ_1- and γ_2-antibody lies in the Fc fragment (32, 33) which appears to be uninvolved in the structure of the antibody-binding site (33, 34).

We have compared precipitin reactions of γ_1- and γ_2-antibodies. It was found that γ_1-antibodies, which are more highly charged at neutral pH, exhibit more marked inhibition of precipitation in antigen excess. That is, the more charged, and therefore more hydrophilic, antibody has a greater tendency to form soluble complexes in antigen excess. When DNP-BGG is used as antigen, the marked insolubility of this relatively uncharged protein predominates and, as has been observed previously (35), no inhibition zone is seen. The differences in behavior in antigen excess emphasize, once again, that nonspecific forces [e g., charge density of the antigen molecule (35), charge density of the antibody molecule (present observations), protein-protein interactions between antibody molecules (36)] play a significant role in the precipitation reaction. It is obvious that the differences in precipitation curves of γ_1- and γ_2-molecules cannot be accounted for by any simple stoichiometric relationships.

The data given in Tables III and IV seem to suggest a rough correlation between the amount of antibody formed, per cent γ_2, and affinity. For example, DNP-BGG elicits the greatest antibody response with the highest per cent of γ_2-antibody and the antibody of highest affinity. However, if the values are examined within any class (antibody induced with a particular carrier) no

correlation exists between the amount of antibody made by a particular animal and the affinity of its antibody. Therefore the apparent parallelism of amount of antibody, electrophoretic class, and association constant may be purely fortuitous.

These experiments show that an animal does not make a unique antibody molecule (or a special population of molecules) upon exposure to DNP antigens regardless of the environment of the DNP determinant. Populations of antibody molecules are formed which, on the average, bind the DNP hapten more or less strongly depending on the carrier molecule used for immunization. This point is further elaborated in the companion paper in which "carrier specificity" is specifically investigated. This variability in anti-DNP combining sites is, of course, superimposed upon the well known heterogeneity of affinity of antihapten antibodies found in sera of individual animals (29, 37, 38).

It is not possible with our present state of knowledge of antibody synthesis to explain the mechanism by which the carrier effects reported here occur, but it is clear that in hapten-protein systems such as that studied here the antigenic determinant encompasses more than the hapten itself (14). In view of this, it is probable that differences in the "exposure," or orientation of the DNP-group, resulting from interaction with adjacent portions of the carrier will affect the configuration of the over-all antigenic determinant and therefore the affinity of the antibody synthesized for the model "homologous" ligand ϵ-DNP-L-lysine. The difference in affinity observed with different carriers must reflect structural differences in the immunoglobulins synthesized. This might involve differences in cell population stimulated by DNP on different carriers. It is of interest to consider that within each carrier molecule the microenvironment of DNP groups differs. In view of this heterogeneity the fact that one can clearly observe carrier effects suggests that some DNP groups act as the predominant antigenic determinant in each case.

SUMMARY

1. Fluorescence quenching has been calibrated, by comparison with equilibrium dialysis, for measurement of antibody-hapten association constants with guinea pig antibody to the 2,4-dinitrophenyl (DNP) determinant. The maximum quenching with all sites occupied which gave best agreement with equilibrium dialysis was found to be 100%.

2. The carrier to which the DNP-hapten is coupled influences significantly the amount, class (γ_1 or γ_2), and affinity of the antibody of DNP specificity made by individual guinea pigs.

3. The affinities of γ_1- and γ_2-antibodies from individual guinea pigs are generally very similar.

4. Precipitin curves with γ_1-antibodies show more marked inhibition of precipitation in antigen excess than do precipitin curves with the less charged

γ_2-antibodies, indicating the importance of nonspecific forces in the precipitation reactions.

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