POLYFUNCTIONAL DINITROPHENYL HAPTENS AS REAGENTS FOR ELICITATION OF IMMEDIATE TYPE ALLERGIC SKIN RESPONSES*

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In a previous study it was shown that coupling 2,4-dinitrophenyl groups to human serum protein yielded a stable conjugate that elicited wheal-and-erythema skin responses in a human subject sensitive to this simple determinant (1). Protein conjugates of this kind are potentially useful for detecting sensitivity in humans to simple determinants, particularly since unifunctional haptens (*i.e.*, those which contain only a single determinant per molecule) can competitively block the action of the corresponding polyfunctional protein conjugates, and thereby establish the specificity of the allergic skin responses with considerable confidence (1). Despite their promise as diagnostic reagents for the detection of sensitivity to simple substances, the widespread use of protein conjugates for testing human populations is, however, potentially hazardous since the intradermal injection of conjugates might well stimulate the formation of antibodies. We have therefore sought to develop a general class of reagents which combines effectiveness in eliciting wheal-and-erythema responses with inability to stimulate antibody formation. The feasibility of preparing reagents which satisfy both of these requirements is suggested by two kinds of immunological experience: (*a*) exclusive of certain protein-reactive reagents, substances of molecular weight less than 5000 do not evoke antibody formation, and (*b*) protein conjugates are effective in eliciting immediate type allergic responses primarily because they are polyfunctional; *i.e.*, because they carry 2 or more determinants per molecule (1, 2). In view of these considerations, we have undertaken to examine a number of substances of graded sizes, carrying a varied number of determinants, in respect to their capacity to evoke

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specific skin responses and to induce antibody formation. In the present paper the principles involved in the design of skin test reagents which meet the requirements elaborated above have been explored through the use of the 2,4-dinitrophenyl determinant as a model. In the accompanying papers, the results obtained with the model system are applied to determinants derived from penicillenic acid and the reagents developed are evaluated in the guinea pig and in man (3, 4).

Materials and Methods

Reagents.—Bovine γ-globulin and human serum albumin were obtained from Armour and Co., Chicago, and Pentex Corp., Kankakee, Illinois, respectively.

Polylysine preparations having an average of 8 and 20 lysyl residues per molecule were a gift of the Kremers-Urban Co., Milwaukee.[1] A third polylysine preparation of average molecular weight 85,000 (about 410 residues per molecule) was obtained from the Pilot Chemical Company, Watertown, Massachusetts. These polylysines are referred to hereafter as poly 8, poly 20, and poly 410, respectively. The values for molecular weight are based on the manufacturers' assays. Succinic anhydride and 2,4-dinitrobenzene sulfonate were obtained from Eastman Organic Chemicals, Rochester, New York. The latter reagent was recrystallized 4 times from water. Chromatographically pure bis-DNP-lysine[2] was obtained from the Mann Co., New York City, and amberlite IRA-400 anion exchange resin from the Fisher Scientific Co., Fair Lawn, New Jersey.

Preparation of Antigens.—DNP-protein conjugates were prepared and characterized as described previously (5). DNP-BγG and DNP-HSA contained, respectively, an average of 58 and 29 DNP groups per protein molecule.[2]

Preparation of Dinitrophenyl-Polylysine 410.—84 mg poly 410 (hydrobromide salt), 28 mg dinitrobenzene sulfonate, and 48 mg K_2CO_3 were dissolved in 10 ml water and were shaken slowly for 12 hours at room temperature. At the end of this period a small amount of insoluble material[3] was removed by centrifugation, and the solution was passed through IRA-400 (Cl^- form) to remove unreacted dinitrobenzene sulfonate and dinitrophenol. The yellow effluent was then dialyzed for 4 days at 4°C against 0.001 M PO_4, pH 7.4. The product had an absorption maximum at 354 mμ. Based on determinations of dry weight, absorbance at 360 mμ, and the molar extinction coefficient for ε-DNP-lysine (17,400 at 360 mμ), it was calculated that the product contained 90 DNP groups per average polylysine molecule; this preparation is referred to below as DNP_{90}poly 410.

Succinylation.—48 mg DNP_{90}poly 410 were reacted with 720 mg succinic anhydride in 120 ml 0.14 M $NaHCO_3$ at room temperature over-night. The product was dialyzed at 4°C for 4 days against 0.001 M PO_4, pH 7.4. The absorbance at 360 mμ per mg of dialyzed material was appreciably lower for the succinylated product than the original DNP_{90}poly 410. This

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[1] We wish to thank Dr. Brian Lees, medical director of the Kremers-Urban Co. for the generous gift of these polylysine preparations.

[2] Abbreviations: DNP is used generically for the 2,4-dinitrophenyl group combined with amino acids or proteins. ε-DNP-lysine, ε-2,4-dinitrophenyl-lysine; DNP-BγG, 2,4-dinitrophenyl-bovine gamma globulin, etc. Subscript numbers in DNP-proteins and DNP-polylysines refer to approximate number of DNP residues per molecule protein or polylysine. HSA is human serum albumin.

[3] When the relative concentration of dinitrobenzene sulfonate was doubled (1 mole dinitrobenzene per 2 lysine residues), a gelatinous yellow precipitate formed, and the supernate contained only a small amount of soluble DNP-polylysine.

change in absorbance corresponded to the addition of 210 succinyl groups per molecule DNP_{90}-poly 410. This figure agreed well with the value of 195 succinyl groups per molecule determined by acidimetric titration.

Preparation of Dinitrophenyl–Polylysines 8 and 20.—50 mg poly 8 and poly 20 (each as the hydrochloride salt) were reacted with 32 and 26 mg dinitrobenzene sulfonate, respectively, in 10 ml 0.025 M K_2CO_3; after 36 hours at room temperature a considerable amount of insoluble material was present in the poly 8 reaction mixture, but the poly 20 preparation was largely soluble. In both cases insoluble material was removed by centrifugation and the supernatants were passed through IRA-400 (Cl^- form). Dialysis was not used for further purification because of the relatively small molecular size of these derivatives.

The total column effluent of DNP–poly 8 (approximately 25 mg) was mixed with 150 mg succinic anhydride and 393 mg $NaHCO_3$ in a total volume of 30 ml. A portion of the column effluent of DNP–poly 20 (approximately 40 mg) was mixed with 150 mg succinic anhydride and 197 mg $NaHCO_3$ in a total volume of 15 ml. After shaking overnight, acidification to pH 2–3 with 2 N HCl yielded quantitative precipitation of both of these polylysine derivatives as judged by the virtual absence of 360 mμ absorbance in the supernatants. The acid insolubility of the materials after reaction with succinic anhydride was in contrast to the behavior of the original DNP-polylysines under similar pH conditions and clearly indicated some degree of succinylation. The acid precipitates were washed 5 times with water and dried. Based on absorbance at 360 mμ per mg dry weight, and acidimetric titration in aqueous solution, it was estimated that the succinylated poly 20 contained approximately 6 to 7 DNP groups per molecule and that more than one-half of the remaining amino groups were succinylated. The succinylated DNP–poly 8 contained approximately 2 DNP groups per molecule. Its solubility in aqueous solution was relatively low, precluding accurate acidimetric titration for succinyl groups.

Antisera.—In comparing various conjugates with respect to immunogenicity, each of the 5 different substances listed in Table II was given to a separate group of 5 guinea pigs. In each animal 1 mg of test material in 0.8 ml complete Freund's adjuvant was distributed among the four foot-pads (6, 5 *c*). 2 weeks later the animals were bled. Sera were cleared and assayed for antibody activity by the ring precipitin test and by passive cutaneous anaphylaxis in guinea pigs (7). In the precipitin assay, the test antigens were DNP-HSA and the succinylated DNP-polylysines.[4]

One pool of rabbit antiserum was prepared by injecting each of 40 rabbits with 5 mg DNP-BγG in complete Freund's adjuvant (0.4 ml per foot-pad). Sera obtained by cardiac puncture 1 month after injection were pooled (pool A). A second pool of rabbit antiserum was prepared by thrice-weekly injections of alum-precipitated DNP-BγG for 4 weeeks, a total of 56 mg DNP-BγG being injected in each rabbit; the first injection each week was subcutaneous, the others, intravenous. Sera were obtained on the 5th, 6th, and 7th days after the last intravenous injection, and were pooled (pool B).

A pool of guinea pig serum was obtained from animals that had been injected once and bled 1 month later; each guinea pig received 1 mg DNP-BγG in 0.8 ml Freund's adjuvant distributed evenly among the foot-pads.

Antibody Purification and Characterization.—Purified anti-DNP antibodies were prepared separately from rabbit serum pools A and B and from the pool of guinea pig antiserum by a method described previously (5 *c*). Association constants for the reaction of the purified antibodies with ε-DNP-lysine were measured by the fluorometric method described by Velick *et al.* (8).

[4] Precipitates appeared when DNP-polylysines were added to normal rabbit serum. Succinylated DNP-polylysines did not form precipitates with normal serum, but did form precipitates with rabbit and guinea pig anti-DNP antiserum.

Passive Cutaneous Anaphylaxis.—250 gm albino guinea pigs were injected with 0.1 ml serum at 4 to 6 sites on their ventral surface (7). 5 to 6 hours later 1 mg of test antigen and 5 mg Evans blue in a total volume of 1 ml were injected intravenously. Just prior to use, the dye-antigen mixture was cleared by centrifugation at about 11,000 *g* for 10 minutes. Animals were sacrificed 15 to 30 minutes later, skinned, and reactions were graded from 0 to 4+ using the criteria recommended by Ovary (7).

When a series of antisera prepared against different putative antigens were being compared (*e.g.*, Table II), single test animals were injected intradermally with one antiserum of each type so that the results could be contrasted in the same animal. An unknown serum was considered devoid of detectable antibody only when the test animal gave a positive response at a site prepared with a standard anti-DNP serum. In evaluating the various DNP derivatives for capacity to evoke passive cutaneous anaphylaxis, the sites were all prepared with a standard antiserum that reacted strongly with DNP-proteins.

Skin tests in human subjects were performed and scored as described elsewhere (1). Some of the skin responses of the human subject (HNE), who is actively sensitive to the DNP group, have also been described in a previous report (1).

Anaphylaxis.—The ability of different DNP conjugates to produce anaphylaxis was evaluated in 250 gm guinea pigs passively sensitized by intraperitoneal injection of 1.6 ml rabbit anti-DNP antiserum, containing 2 mg anti-DNP antibody as measured by precipitation with DNP-BγG. The animals were challenged 22 hours later by intravenous injection of 0.3 μeq DNP in the form of bis-DNP-lysine or succinylated DNP-polylysines.

RESULTS

The effectiveness of various polyfunctional DNP derivatives in eliciting passive cutaneous anaphylaxis is summarized in Table I. In contrast to the efficacy of bis-DNP-lysine, the lysine derivative with 1 DNP group per molecule (ϵ-DNP-lysine) was shown previously not to be effective; ϵ-DNP-lysine can, in fact, specifically inhibit this allergic response (9, 10).

Results bearing on the immunogenicity of DNP derivatives are summarized in Table II. Of the five antigens tested, only DNP-BγG induced the formation of detectable serum antibody as indicated by passive cutaneous anaphylaxis and ring precipitin tests.

The significance of the negative results with sera prepared against bis-DNP-lysine and the DNP polylysines depends, of course, on the sensitivity of passive cutaneous anaphylaxis as an assay. Inasmuch as published figures (7) on the sensitivity of this assay deal with different systems and are of questionable relevance here, sensitivity was determined with a mock antiserum prepared by dissolving purified guinea pig anti-DNP antibody in normal guinea pig serum. From the experimental details and the results given in Table III, it is apparent that a serum could contain as much as 4 μg anti-DNP antibody per ml and still fail to give passive cutaneous anaphylaxis. It is, however, somewhat misleading to characterize the sensitivity of this assay with a single figure because of its probable dependence not only on the amount of antibody injected in the skin but on the antibody's affinity for the test antigen. That is, less antibody of high affinity is likely to be required for a positive skin response than in the case of low affinity antibody. Data bearing on the influence of antibody affinity on skin-test responses are given below.

Before testing human subjects systematically with bis-DNP-lysine and the DNP-polylysines, preliminary skin tests were performed in a few normal

TABLE I

Efficacy of Various Dinitrophenyl Derivatives as Elicitors of Passive Cutaneous Anaphylaxis in the Guinea Pig

Skin sites prepared with antiserum* from guinea pig No.	Dinitrophenyl derivative used to elicit response‡		
	bis-DNP-lysine	DNP-polylysine 410	DNP-HSA
1	2+	2+	4+
2	2+	1+	4+
3	1+	1+	4+
4	4+	1+	4+
5	3+	3+	4+

* Antisera prepared with a single injection of $DNP_{58}B\gamma G$ in Freund's adjuvant. 1 mg of each derivative was given intravenously, amounting to 2000, 900, and 386 mμeq DNP for bis-DNP-lysine, DNP–polylysine 410, and DNP-HSA, respectively. A solution of these derivatives with an absorbance at 360 mμ of 0.174 has 10 mμeq amino-substituted DNP per ml. The DNP–polylysine 410 was not succinylated; it precipitated with both the Evans blue dye and with normal guinea pig serum. Responses were scored as recommended by Ovary (7).

‡ Skin sites prepared by injection of sera from guinea pigs injected with bis-DNP-lysine in Freund's adjuvant (see Table II) did not react to intravenous injections of the derivatives listed.

TABLE II

Antibody Formation Induced by Various Dinitrophenyl Derivatives as Indicated by Passive Cutaneous Anaphylaxis in the Guinea Pig

Serum obtained from guinea pigs injected with*	Skin responses in guinea pig No.‡				
	1	2	3	4	5
bis-DNP-lysine	0	0	0	0	—
DNP_2poly 8, succinylated	0	0	0	0	0
DNP_7poly 20, "	0	0	0	0	0
DNP_{90}poly 410	0	0	0	0	0
$DNP_{58}B\gamma G$§	4+	4+	4+	4+	4+

* Each guinea pig received 1 mg of one of the substances listed in Freund's adjuvant (see Materials and Methods). Each response recorded corresponds to an individual animal's serum. Each vertical column represents a single guinea pig used to assay 5 antisera at 1 time.

‡ Skin responses were elicited with 1 mg $DNP_{29}HSA$ (see Materials and Methods).

§ All sera from guinea pigs injected with $DNP_{58}B\gamma G$ gave strongly positive ring precipitin tests, using 50 μg antigen per ml. All other sera gave negative ring precipitin tests.

human beings. Bis-DNP-lysine was innocuous, but intradermal injection of the DNP-polylysines (10 to 11 mμeq DNP per site) was followed promptly by urticaria. This non-specific response is not surprising since unsubstituted

polylysines are known to produce reactions in mammalian skin similar to those induced by histamine, presumably by virtue of their strongly positive charge (11). When polylysines are completely substituted with DNP groups, so as to

TABLE III

*Sensitivity of Passive Cutaneous Anaphylaxis as an Assay for Anti-Dinitrophenyl Antibody Diluted in Guinea Pig Serum and in Saline**

Concentration of antibody injected in skin sites	Responses to	
	Antibody diluted with normal guinea pig serum	Antibody diluted with saline
μg/ml		
50	4+	4+
25	4+	4+
15	4+	4+
10	3+	4+
5	2+	4+
3	0	3+
2	0	1+
1	0	0
0.5	0	0
0.3	0	0
0.2	0	0
0.1	0	0

* 52 mg portions of lyophilized purified guinea pig anti-DNP antibody (see Materials and Methods) was taken up in 24 ml normal guinea serum or in 24 ml 0.15 M NaCl–0.01 M PO_4, pH 7.4. The 2 solutions were clarified by centrifugation at ~11,000 *g* for 15 minutes. The absorbance of the saline solution at 278 mμ corresponded to a γ-globulin concentration of 1.61 mg per ml. By quantitative precipitin assay, using $DNP_{58}B\gamma G$ as test antigen, 1.43 mg antibody per ml were found for both the saline and the serum solutions. The serum and saline solutions were diluted with normal guinea pig serum and phosphate-saline, respectively. Skin sites were prepared with 0.1 ml of each dilution and responses were evoked with 1 mg $DNP_{29}HSA$ given intravenously (see Materials and Methods), using duplicate animals. The antibody concentrations listed are based on the amount of antibody recoverable by specific precipitation in the original undiluted stock solutions.

By fluorometric titration (8), the association constant for the interaction of the purified guinea pig antibody with ε-DNP-lysine was 2×10^8 liters per mole at 28°C, at an antibody concentration where 65 per cent of the antibody sites were titrated (assuming 90 per cent purity, antibody molecular weight 160,000, and 2 combining sites per antibody molecule).

remove all cationic sites, they are insoluble. When only partially substituted so as to retain water solubility, they remain strongly cationic and, as noted above, produce urticaria non-specifically. Satisfactory test reagents were, however, obtained by succinylation of the DNP-polylysines (Table IV, tests 7, 8, 10, 11). As shown in Table IV, all of the succinylated DNP-polylysines tested were effective in eliciting wheal-and-erythema responses in the DNP-

sensitive human subject; the responses were similar in size to those evoked with a comparable amount of DNP-HSA.

In contrast to its effectiveness in eliciting passive cutaneous anaphylaxis in the guinea pig, bis-DNP-lysine was apparently inert in the DNP-sensitive human subject (Table IV, tests 1 and 2). It seemed possible that the variable effectiveness of bis-DNP-lysine in eliciting skin responses might reflect differences in affinities of the antibodies in these 2 different test situations. This

TABLE IV

Efficacy of Various Dinitrophenyl Derivatives in Elicitation of Wheal-and-Erythema Responses in Human Subjects

Subject	Test No.	Intradermal injection*		Response
		Substance	Quantity as DNP‡	
			mμeq	
H.N.E.	1	bis-DNP-lysine	4	0
(known sensitivity	2	"	34	0
to dinitrophenyl)	3	$DNP_{29}HSA$	4	4+
	4	DNP_2poly 8, succinylated	11	4+
	5	DNP_7poly 20, "	10	4+
	6	DNP_{90}poly 410, "	10	4+
M. K.	7	DNP_2poly 8, succinylated	11	0
(no known sensitivity	8	DNP_7poly 20, "	10	0
to dinitrophenyl)	9	DNP_{90}poly 410, not succinylated	10	4+
	10	" , succinylated	10	0
	11	" , "	21	0

After skin tests 4, 5, and 6, and a repetition of 6, the subject (HNE) experienced a systemic reaction (urticaria and substernal distress).

* 0.05 to 0.1 ml injected intradermally.

‡ 10 mμeq per ml of NH_2-substituted DNP corresponds to an absorbance at 360 mμ of 0.174.

possibility was evaluated by comparing the response to bis-DNP-lysine at human skin sites passively sensitized with 2 lots of purified rabbit antibody which differed in their affinity for the DNP determinant. Lot 1, which had been prepared from pool A antiserum (obtained by immunizing rabbits with 5 mg of DNP-BγG in Freund's adjuvant), had relatively high affinity for ε-DNP-lysine ($K_A = 1.6 \times 10^8$ liters per mole; Fig. 1). Lot 2, which had been prepared from pool B antiserum (obtained by injecting rabbits intravenously with 56 mg alum-precipitated DNP-BγG), had a much lower average affinity for ε-DNP-lysine ($K_A = 1.3 \times 10^6$ liters per mole; Fig. 1). It is important to emphasize that the two preparations were obtained by the same purification

procedure (5 *c*), and were equally precipitable by DNP-BγG (88 per cent for lot 1 and 92 per cent for lot 2).

Skin sites prepared with the antibody of lower affinity failed to give a wheal-and-erythema response with concentrations of bis-DNP-lysine which were sufficient to give strongly positive responses in sites prepared with an equal quantity of high affinity antibody (Table V). Nonetheless, sites prepared

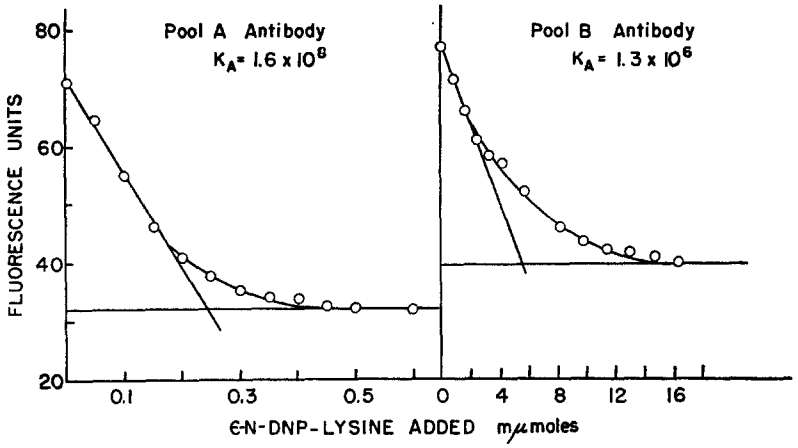


FIG. 1. Association constants for binding of ϵ-DNP-lysine by purified anti-DNP antibodies. In titrating pool A (Freund's adjuvant prepared), antibody concentration was 41 μg per ml. In titrating pool B (alum-precipitated, intravenous preparation), antibody concentration was 649 μg per ml. Solvent, 0.15 M NaCl–0.01 M phosphate, pH 7.4. Temperature, 30°C. Titrations were performed with 1.0 ml protein solution. With pool A, the end point of titration with hapten (0.245 mμmoles) corresponds to 54 per cent of antibody sites, and with pool B, the end point of titration with hapten (5.54 mμmoles) corresponds to 72 per cent of antibody sites (on the basis of the antibody being 90 per cent pure, taking antibody molecular weight to be 160,000 and the number of binding sites per molecule to be 2; see reference 8). Fluorescence is expressed in arbitrary units. Association constants are expressed in liters per mole.

with the low affinity antibody preparation gave strongly positive responses with DNP-HSA (Table V, subject J. P.). As expected, ϵ-DNP-lysine inhibited the response to bis-DNP-lysine in sites which would otherwise have responded (Table V, subjects E. S. and B. F.).

The relative competency of various DNP conjugates in evoking systemic anaphylaxis was not of major concern in this work, and relatively few animals have been studied. It seems clear, however, that even small bivalent conjugates can evoke anaphylaxis as evidenced by results with bis-DNP-lysine which produced anaphylaxis in each of the 5 sensitized animals tested (2 fatal, 3 severe), and completely negative results with 6 control animals. In view of the efficacy of bis-DNP-lysine, the several preparations of succinylated DNP-

polylysines were not tested systematically. It may be noted, however, that the succinylated DNP-polylysines produced anaphylaxis in each of 6 animals tested (4 fatal, 2 non-fatal); only 2 control animals were tested with these derivatives and each failed to respond.

TABLE V

Effect of Antibody Affinity on Capacity of Bis-DNP-Lysine to Elicit Passive Wheal-and-Erythema Responses in Human Subjects

Subject	Purified antibody used to prepare skin sites		Sites tested with		Response
	Source*	Amount injected	Substance	Quantity as DNP	
		μg		mμeq	
E. S.	Pool A	130	bis-DNP-lysine	170	++
		130	"	17	++
		130	bis-DNP-lysine + ε-DNP-lysine	17 230	0
		130	bis-DNP-lysine	2	++
B. F.	Pool A	130	bis-DNP-lysine	170	++
		130	"	2	++
		130	bis-DNP-lysine + ε-DNP-lysine	17 230	0
		130	$DNP_{38}HSA$	7	++
J. P.	Pool A	130	bis-DNP-lysine	2	++
	Pool B	130	"	2	0
		130	"	17	±
		130	$DNP_{29}HSA$	2	++

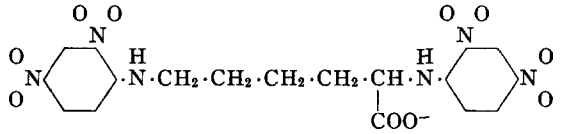
* Pool A antibody was prepared with $DNP_{58}B\gamma G$ in Freund's adjuvant, and pool B antibody was prepared with the same antigen, but alum-precipitated and given intravenously (see Materials and Methods). See Fig. 1 for the respective association constants with ε-DNP-lysine.

DISCUSSION

A number of previous studies have suggested that the minimal number of determinants per molecule required for effective elicitation of anaphylaxis is 2 (2 *b*, 12). Since bis-DNP-lysine is anticipated to be incapable of inducing antibody formation, it could have been expected at the outset that this substance might be the simplest one that could meet the objectives of the present study. However, while bis-DNP-lysine elicited systemic and passive cutaneous anaphylaxis in guinea pigs sensitized with purified rabbit anti-DNP antibody (Table I), it failed to elicit a wheal-and-erythema response in a human subject sensitive to the DNP determinant (Table IV). The likelihood that the variable

effectiveness of bis-DNP-lysine in these 2 different situations arose from variations in average affinity of the different antibody populations involved is strongly suggested by the observation that human skin sites prepared with rabbit antibodies of relatively low affinity failed to react to bis-DNP-lysine while sites prepared with rabbit antibodies of high affinity reacted strongly (Table V).

The foregoing results are of general significance in demonstrating the importance of affinity in determining the biologic activity of antibodies. More specifically, in the present instance, the dependence on antibody affinity of the skin test response to bis-DNP-lysine may be interpreted on the basis of the asymmetric disposition of the DNP groups in this molecule:



As reported previously (8), purified anti-DNP antibodies as prepared herein have a strong affinity for ε-DNP-lysine ($\Delta F°$, −11 kcal per mole) and much less affinity for 2,4-dinitrophenol ($\Delta F°$, −7 kcal per mole). These antibodies also have a much lower affinity for DNP-glycine than for ε-DNP-lysine (5 *c*). It seems likely that the unusually high affinity for ε-DNP-lysine arises from apolar (13) interaction of the $(CH_2)_4$ group of the lysine moiety with appropriate groups of the antibody-combining region. On this basis, anti-DNP antibodies should have a higher affinity for the ε-DNP group than for the α-DNP group of bis-DNP-lysine. Moreover, combination of 1 antibody molecule with the ε-DNP group could be expected to hinder, sterically and electrostatically, the attachment of a 2nd antibody molecule to the α-DNP group, and thereby accentuate even more the functional dissimilarity of the 2 determinant groups of bis-DNP-lysine. We visualize, therefore, that while with antibody populations of high affinity both of the DNP groups of bis-DNP-lysine could be simultaneously bound by antibody molecules, with antibody populations of lower affinity the α-DNP group might be hardly capable of reaction, *i.e.*, bis-DNP-lysine can behave as a bifunctional molecule with antibodies of high affinity, but with antibodies of low affinity this substance appears to have only 1 functional site, undoubtedly the ε-DNP-NH-$(CH_2)_4$ group. Regardless of the precise interpretation for the non-uniform effectiveness of bis-DNP-lysine, it is clear that this substance, and probably also other comparable small bifunctional molecules, are not dependable skin-test reagents in screening for sensitivity to simple determinants.

In contrast to bis-DNP-lysine, the DNP-substituted polylysines were regularly effective in eliciting wheal-and-erythema responses (Table IV), although it was necessary to succinylate these polymers in order to eliminate their non-

specific reactivity in skin. Even the smallest conjugated polylysine (succinylated), with an average of 2 DNP residues in a polymer having an average of 8 lysine residues, was effective under circumstances where bis-DNP-lysine was not (Table IV). That this conjugate, and the more extensively substituted larger polylysines (all succinylated), were more effective than bis-DNP-lysine may be ascribed to the probability that their DNP substituents are distributed at random and are likely to be sufficiently distant from one another to permit a single molecule of conjugate to accommodate 2 or more antibody molecules at the same time. Moreover, in the conjugated polylysines virtually all DNP substituents are combined with lysine ϵ-amino groups, thereby affording the possibility for antibody to combine strongly with any of the individual determinants. The latter consideration was, in fact, one of the main reasons for choosing polymers made of lysine residues as carriers for the DNP determinant. An additional reason for choosing polylysine as a carrier is provided by the following considerations: polyamino acids made from a single kind of amino acid (homopolymers) are likely to be incapable of inducing antibody formation. While there is actually some conflicting evidence concerning the immunogenicity of such homopolymers it is clear that their capacity to stimulate antibody formation is feeble at best (14). On the other hand certain heteropolymers, made from 2 or more different amino acids, are capable of inducing antibody formation (15). Heteropolymers have, therefore, been avoided in the present work even though some of them would have been otherwise desirable because of their solubility and charge characteristics. The inability of the DNP-substituted polylysines to induce anti-DNP antibody formation is consistent with the negligible immunogenicity of unsubstituted polylysine (14, 16), and is in agreement with the observations of Sela and Haurowitz (17) who found that polytyrosines substituted with azobenzene arsonate groups were incapable of inducing the formation of anti-benzene arsonate antibodies.

DNP-polylysines bear a number of interesting resemblances to purified pneumococcal polysaccharides. Both of these polymers combine effectively with antibody *in vivo* and *in vitro* and elicit a variety of immediate allergic responses (18, 21). The purified pneumococcal polysaccharides can, however, stimulate a significant antibody response (19). Although guinea pigs injected with DNP-polylysines failed to form detectable amounts of antibody, the passive cutaneous anaphylaxis assay used to evaluate their immunogenicity is only moderately sensitive; *i.e.*, it can detect antibody only at concentrations greater than 4 μg per ml. In respect to the potential use in man of substituted polylysines analogous to DNP-polylysine (4) it is necessary to recall that pneumococcal polysaccharides exhibit greater antigenic power in man than in some rodents (19, 20). As a further precautionary note in respect to the potential use of the substituted polylysines as diagnostic reagents in humans it should be emphasized that the present study was mostly concerned with the

induction of antibody formation *de novo*. It is possible that in individuals already sensitized, the polylysine derivatives may provide a significant stimulus to additional antibody formation. Further experience with this class of compounds will be required to evaluate the latter possibility.

SUMMARY

Dinitrophenyl derivatives of differing molecular weights and degrees of substitution have been contrasted with respect to their ability to elicit immediate type allergic responses and their capacity to induce antibody formation in the guinea pig. In contradistinction to dinitrophenyl-proteins, bis-DNP-lysine and DNP-polylysines (including a 100,000 molecular weight derivative) failed to induce antibody detectable by guinea pig passive cutaneous anaphylaxis. Dinitrophenyl-polylysines evoked urticarial responses non-specifically, but after succinylation were about as effective as dinitrophenyl-proteins in eliciting specific cutaneous reactions. An important factor influencing the effectiveness of bis-DNP-lysine in evoking specific wheal-and-erythema responses is antibody affinity for the dinitrophenyl-lysyl determinant.

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