The Nature of the Antigen-Antibody Complexes Initiating the Specific Wheal-and-Flare Reaction in Sensitized Man

BERNARD B. LEVINE and ANTHONY P. REDMOND

From the New York University School of Medicine, Department of Medicine, New York

ABSTRACT To study the nature of the antigen-antibody complexes which initiate the specific wheal-and-flare (W & F) reaction in sensitized man, a homologous series of bivalent, oligovalent, and multivalent benzylpenicilloyl (BPO) haptens were quantitatively compared for their effectiveness in eliciting W & F in BPO-sensitized human subjects.

A series of seven divalent haptens were capable of eliciting W & F, but these generally were not maximally effective elicitors. Of the divalent haptens, those with separation chains of 8 or 13 A were the most effective. Of the oligovalent haptens, maximal effectiveness was attained with BPO_6-$lysine_7$, and not with BPO_3-$lysine_3$ or BPO_4-$lysine_4$, i.e., haptens which are 6- 3- and 4-valent, respectively, from a chemical point of view. However, evidence was obtained from quantitative precipitation experiments which indicated that BPO_6-$lysine_7$ functions as a trivalent hapten immunologically, i.e., capable of binding three antibody molecules per mole hapten. Large molecular–sized haptens with immunological valences of 7 or 12, but in which the haptenic groups were widely separated, were comparatively ineffective elicitors of W & F. In individual subjects, threshold W & F reactions were obtained with equimolar concentrations of the differently sized divalent, oligovalent, and multivalent haptens.

The results demonstrate that for maximally effective elicitation of W & F by haptens, trivalency with optimal distances of separation of haptenic groups is necessary and sufficient. These results indicate the requirement for the formation of a high energy complex of two or three membrane-fixed skin-sensitizing antibody molecules closely bridged together by the elicitor hapten as the initiator of the W & F reaction.

Dr. Bernard B. Levine is a Career Scientist of The Health Research Council of the City of New York.

Received for publication 5 September 1967 and in revised form 25 October 1967.

INTRODUCTION

As an approach to the study of the nature of the antigen–antibody complexes that initiate the specific wheal-and-flare reaction (W & F), we have prepared and quantitatively compared the abilities to elicit W & F of a homologous series of benzylpenicilloyl (BPO) haptens in BPO-sensitized patients.

In a previous paper (1) it was reported that, univalent haptens were ineffective at eliciting W & F, a bivalent hapten was variously effective, and that multivalent haptens of widely differing molecular sizes were equally effective, when compared on an equimolar concentration basis but not on an equal-weight concentration basis. By contrast, in specific precipitation and the active Arthus reaction in guinea pigs the bivalent hapten was virtually ineffective, whereas the differently sized multivalent haptens were equally effective when compared on an equal-weight concentration basis, but not on an equimolar concentration basis (2). These findings were interpreted as indicating that W & F is initiated by the simple bridging of a small number of "fixed" (to a membrane surface) skin-sensitizing antibody (SSA) molecules by the antigen, and not by the formation of cross-linked

lattice complexes (see references 1 and 2 for the supporting arguments). Similar findings and interpretations were made for passive cutaneous anaphylaxis (PCA) in the guinea pig (2, 3).

The present study is concerned with the following questions: (*a*) Can a divalent hapten become a maximally effective elicitor of W & F if its size or structural rigidity is made optimal? (*b*) If a divalent hapten is not a maximally effective elicitor, what is the minimum valence necessary for a hapten to elicit maximally intense W & F? (*c*) What is the effect of the distance between haptenic groups on the effectiveness of the haptenic elicitor? In this study, we compared a group of six bivalent haptens of different sizes and rigidities, as well as various oligovalent and multivalent BPO-haptens, for their effectiveness in eliciting W & F reactions. The results are best interpreted as indicating that a trivalent hapten (whose haptenic groups are separated by an optimal distance) is generally necessary and sufficient to elicit maximally intense W & F reactions.

METHODS

Materials

Crystalline potassium benzylpenicillin was donated by Bristol Laboratories, Syracuse, N. Y. 1,3-propane-diamine was purchased from Eastman Organic Chemical Co., Rochester, N. Y. Hexamethylenediamine (1,6-diaminohexane) and nonamethylenediamine (1,9-diaminononane) were donated by E. I. duPont, Industries, Wilmington, Del.; *N,N*-bis(3-aminopropyl)sebacamide was donated by Union Carbide Chemical Co., So. Charleston, W. Va.; dodecamethylenediamine (1,12-diaminododecane) and 4,4′-bis(3-aminopropyl)-biphenyl·2HCl were donated by Wallace Laboratories, Cranbury, N. J.

Tri-L-lysine·HCl (*α*) (Lot M-1762) and tetra-L-lysine·HCl (*α*) (Lot M-2190) (Lys_3 and Lys_4) were purchased from Cyclo Chemical Corp., Los Angeles, Calif. Lys_3 gave an intense Lys_3 ninhydrin positive spot and a faint spot corresponding to Lys_5 on paper chromatography (Fig. 1). The Lys_7·HCl preparation was synthesized by Cyclo Chemical Corp. (Lot M-2307). Paper chromatography [1] showed it to be composed mainly of $Lys_{6,7}$, and Lys_8 and to contain a small amount of Lys_5 (Fig. 1). After prolonged chromatography a small amount of ninhydrin positive material remained at the origin, about 5–10% of the total color intensity, as estimated visually. Column chromatography of the Lys_7 preparation on carboxymethyl cellulose (5) was performed by Dr. H. Sober of the National Institutes of Health, Bethesda, Md.,[2] and showed mainly the 6- 7- and 8-mers and a small amount (perhaps 10%) of high molecular weight material. The elution pattern of the high molecular weight impurity indicated it to be higher than the 40-mer. There was no ninhydrin positive material eluted between the 8-mer and the 40-mer.

Poly-L-lysine hydrobromide salt of average degree of polymerization of 535 (Lot L-22), PLI_{535}, was purchased from Pilot Laboratories, Watertown, Mass. The average degree of polymerization was calculated from the measurement of specific viscosity in 0.2 M NaCl, pH 3.0 (per manufacturer's analysis).

Bivalent BPO-haptens. These were prepared by the reaction of the alkyl or aromatic amines with an excess of potassium benzylpenicillin (KPG) in water at alkaline pH (6). BPO_2-PD, BPO_2-HMD, BPO_2-NMD, and BPO_2-APD [3] were prepared by reaction of the amines with 2–3 molar equivalents of KPG in aqueous solution on a pH-stat, pH 11.6, at 25°C for 1 hr with pH maintained at 11.6 by additions of 1 N NaOH. The haptens were precipitated from the reaction solution by acidification to pH 3.6 with 1 N HCl, and were gathered and washed on a sintered glass funnel, dissolved in H_2O at pH 10–11 by additions of 1 N NaOH, then reprecipitated at pH 3.6, washed thoroughly with cold water, and dried in a lyophilizer. In some preparations the resulting white powder was further purified by being dissolved in dry dioxane, precipitated with dry ether, washed with ether, and dried in vacuum. BPO_2-DMD and BPO_2-DAPS [3] were prepared by the reaction of 1 g of the amine with 4 molar equivalents of KPG in 120 ml of ethanol: water (1:3 v/v) at pH 10.8 for 2.5 hr. The reaction solution was adjusted to pH 7, some precipitate was removed by filtration, and the divalent hapten was precipitated from the clear reaction solution by acidification with 1 N HCl to pH 3.5. The white precipitate was washed with cold water, dissolved in water at pH 10–11, and reprecipitated at pH 3.5. The precipitate was then gathered on a sintered glass funnel, washed seven times with cold water, and dried by lyophilization. The resulting white powder was then precipitated from dioxane with dry ether, washed with ether, and dried under vacuum. BPO_2-DDS was prepared by coupling 2 moles of mono-BPO propylenediamine (BPO_1-PD) (BPO-NH-$(CH_2)_3$-NH_2) to 1 mole of difluorodinitrodiphenylsulfone, DPS, (General Biochemical Co., Cleveland, Ohio). BPO_1-PD was prepared by reaction of 10 molar equivalents of propylenediamine with 1 equivalent of benzylpenicillin in aqueous solution for 1 hr. The reaction solution was lyophilized. BPO_1-DP was extracted with dimethylformamide and precipitated with dry ether, washed with ether, and dried under vacuum. 4 equivalents of BPO_1-DP was then reacted with 1 equivalent of DPS in dimethylformamide solution by incubating at 25°C for 30 min. The reaction solution was mixed with 5 volumes of water, brought to pH 3.6 with 1 N HCl, and the precipitate was collected and washed on a sintered glass funnel. The precipitate was dissolved in water at pH 11,

[1] Paper chromatography was done on No. 3 Whatman paper with the solvent system, described by Waley and Watson (4), modified by substitution of *n*-propanol for *n*-butanol.

[2] We thank Dr. H. Sober for performing these analyses.

[3] See Table II for chemical structures.

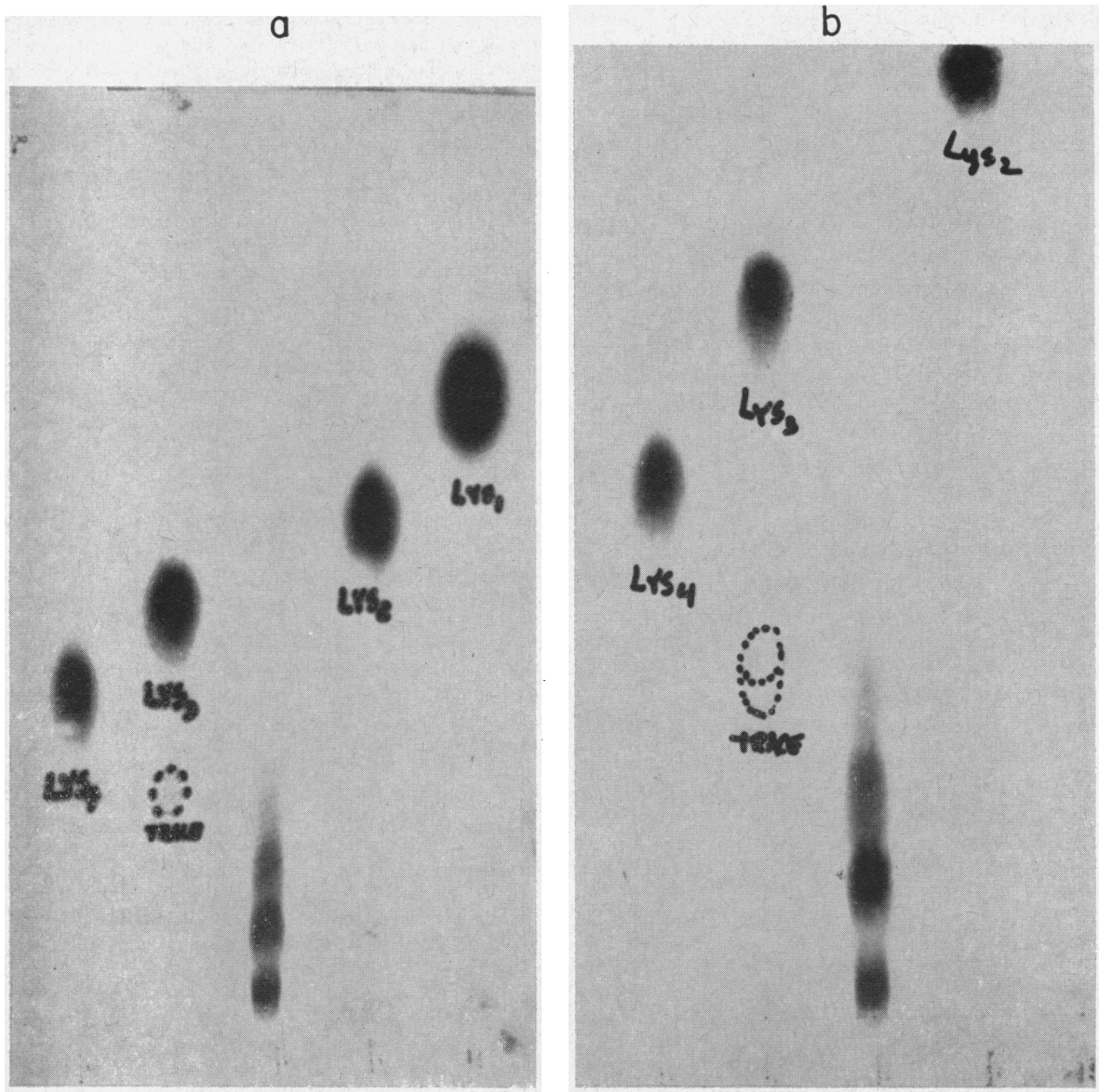

FIGURE 1 Descending paper chromatography of 50 μg samples of lysine, dilysine (Lys_2), trilysine (Lys_3), quadralysine (Lys_4), and the heptalysine (Lys_7) preparation (as HCl salts) on Whatman No. 3 paper with the modified Waley-Watson solvent (footnote 1). 1 *a*, 2 days and 1 *b*, 5 days of chromatography.

reprecipitated at pH 3.6, washed thoroughly with water, and dried in vacuum. The BPO_2-DDS powder was finally slurried in dioxane, precipitated with dry ether, washed with ether, and dried under vacuum. Proof of structure of the divalent haptens is based upon the known benzylpenicilloylation reaction of amines in alkaline aqueous solutions (6); the proper elemental analyses are listed in Table I, as well as the ability of these compounds to rearrange in the presence of *p*-chloromercuribenzoate to form products with intense absorbtion bands at λ_{max} 285 mμ (6), benzylpenamaldates, (Table I). For BPO_2-HMD, molecular weight determination by vapor pressure–depression in pyridine was 788 (calculated: 784, anhydrous). Elemental analyses and vapor pressure–depression determinations were performed by Schartzkopf Laboratories, Woodside, N. Y.

Oligovalent haptens—BPO_3-Lys_3, BPO_4-Lys_4, and BPO_6-Lys_7. 50 mg of the amine hydrochloride was reacted with 5 equivalents of benzylpenicillin per equivalent of ε-NH_2 in 50 ml of water, pH 11.5, for 90 min at 25°C by additions of 1 N NaOH from a pH-stat. The BPO-haptens were precipitated from the reaction solution

TABLE I

Bivalent Haptens

Hapten*	Empirical formula	Elemental analysis						Eм at 285‡
		Predicted			Found			
		C	H	N	C	H	N	
BPO_2–PD	$C_{35}H_{46}O_8N_6S_2 \cdot 2H_2O$	54.0	6.48	10.8	54.1	6.74	11.0	42,500
BPO2–HMD	$C_{35}H_{52}O_8N_6S_2 \cdot H_2O$	56.9	6.74	10.5	56.7	6.90	10.5	43,000
BPO_2–NMD	$C_{41}H_{58}O_8N_6S_2 \cdot H_2O$	58.4	7.18	10.0	58.7	7.19	10.0	42,600
BPO_2–DMD	$C_{44}H_{64}O_8N_6S_2 \cdot H_2O$	59.6	7.48	9.48	59.7	7.78	9.73	40,800
BPO_2–DAPS	$C_{48}H_{70}O_{10}N_8S_2 \cdot H_2O$	57.7	7.26	11.2	57.8	7.59	11.0	54,000
BPO_2–ADP	$C_{50}H_{62}O_8N_6S_2 \cdot H_2O$	62.9	6.77	8.80	62.5	6.70	8.90	42,000
BPO_2–DDS	$C_{50}H_{60}O_{14}N_{10}S_3 \cdot H_2O$	52.7	5.50	12.4	52.7	5.73	12.6	42,000

* For chemical structures, see Table II.

‡ After reaction with *p*-chloromercurobenzoate [penamaldate assay, (6)].

by acidification to pH 3.6 with 1 N HCl, washed five times with cold water, redissolved in water at pH 10–11, reprecipitated at pH 3.6, washed, and finally dried by lyophilization. The products were white powders and the yields were about 50–75% of theoretical. For analysis, the powders were dissolved in 0.05 M phosphate buffer at pH 8–8.5; the solutions were assayed for BPO concentration by the penamaldate method (6) and for lysine N by the micro-Kjeldahl method, with correction for nitrogen contribution of the BPO groups. Triplicate assays were done; mean deviation was ± 5%. BPO groups per mole hapten were 3.1 for BPO_3-Lys_3, 4.1 for BPO_4-Lys_4, and 6.2 for BPO_6-Lys_7. Titration curves (haptens dissolved in water at pH 11) showed 1 equivalent of buffering around pH 8.2 per mole hapten for BPO_3-Lys_3 and BPO_4-Lys_4 (corresponding to a free-terminal α-NH_2 group), and for BPO_6-Lys_7 1 equivalent of buffering around pH 10.5 per mole hapten (corresponding to one free ϵ-NH_2 group).

Multivalent haptens. Multivalent haptens were prepared by reaction of PLL with penicillin in alkaline aqueous solution followed by succinylation of free amino groups as described previously (7). BPO_{15}-$PLL_{20}S$ has been described previously (1, 2). The mole ratios of penicillin to ϵ-NH_2 used to prepare the conjugates were 3.0, 0.03, 0.05, 0.1, 0.3, and 1.0, for BPO_{135}-$PLL_{286}S$, BPO_7-$PLL_{535}S$, BPO_{12}-$PLL_{535}S$, BPO_{24}-$PLL_{535}S$, BPO_{52}-$PLL_{535}S$, and BPO_{139}-$PLL_{535}S$, respectively. Methods of purification and of assay have been described previously (7).

Antisera. Rabbit anti-BPO sera Pool 38 and Pool 39B were prepared by immunization of groups of seven rabbits with 0.5 mg of BPO_{35}-BGG (for Pool 38) or with BPO_{70} bovine fibrinogen (for Pool 39B) in complete Freund's adjuvant, injected into the foot pads; rabbits were boosted with 5 mg of the conjugate, injected i.v. on day 35, and bled on the 42nd, 43rd, and 44th days. Pools 38 and 39B contained 1.90 and 1.42 mg/ml anti-BPO antibody protein precipitable by BPO_{130}-$PLL_{535}S$.

Partially absorbed Pool 38. 85 ml of serum was absorbed with 170 μg of BPO_{15}-$PLL_{20}S$. Pool 38 (abs) contained anti-BPO antibody protein, 1.40 mg/ml, precipitable by BPO_{130}-$PLL_{535}S$.

Quantitative precipitin analyses. Duplicate samples of 1.00 ml of serum were assayed. Hapten–serum mixtures were incubated at 37°C for 1 hr and at 4°C for 4 days. Precipitates were washed three times with ice cold saline, air-dried, and dissolved in 0.1 M NaOH for spectrophotometric analysis. ϵ 1% for rabbit gamma globulin kept in NaOH solution at 25°C for 45 min was 15.8 at λ_{max} 290 mμ. Corrections for optical contribution of haptens were made. Duplicates were in good agreement; mean deviation from mean, ± 3%.

Patients were from the wards of Bellevue Hospital. They ranged from 22 to 80 yr, of both sexes, and were in generally good physical condition. Most had past histories of allergic reaction to penicillin.

Skin-testing methods

Haptens were diluted in TBS-EDTA (0.14 M NaCl, 0.02 M tris-(hydroxymethyl)-aminomethane, 10^{-4} M ethylenediamine tetraacetic acid, at pH 8.2) and sterilized by filtration through Millipore HA filter membranes (washed first by injecting TBS and hapten solution through them). Hapten solutions were generally stored frozen, or for short periods of time, at 4°C. Disposable syringes and needles were used. The posterior-lateral aspects of the arms (7 cm below acromion–10 cm above the olecranon) were used for skin testing. Test sites placed in horizontal rows were found to be of equal reactivity.

Reagent volumes of approximately 0.005 ml each were carefully injected i.d. This was estimated by comparing bleb sizes of volumes injected from disposable syringes with those prepared by injection for a quantitative microsyringe (Hamilton Co., Whittier, Calif.). With experience, volumes of 0.005 ml could be injected reproducibly. Each test was done in triplicate or quadruplicate. Test sites were read after 15 min for wheal-and-flare reaction. Negative tests were blebs of 1–2 mm diameter of indistinct outline and without surrounding erythema. Positive tests were typical wheals of sharp and irregular

TABLE II

Comparative Effectiveness of Bivalent Haptens in Wheal-and-Flare Reactions

Bivalent hapten (BPO-NH-X-NH-BPO) X	Abbreviation	Length‡ of X	Average wheal diameters*													
		Patients.....	E. B.		P. S.		L. M.		J. M.		G. C.		T. P.		C. A.	
			Molar concentrations of haptens.....													
			10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}
		A	*mm*		*mm*		*mm*		*mm*		*mm*		*mm*		*mm*	
$-(CH_2)_3-$	BPO_2–PD	4.5	2	3	3	4	2	4	2	3	2	5	3	3	3	3
$-(CH_2)_6-$	BPO_2–HMD	8.5	5	7	5	6	4	6	5	9	5	7	5	9	5	9
$-(CH_2)_9-$	BPO_2–NMD	13	5	7	5	6	4	6	5	9	5	7	5	9	5	9
$-(CH_2)_{12}-$	BPO_2–DMD	17	3	6	4	5	4	5	5	8	5	7	4	5	5	7
$-(CH_2)_3-NH-CO-(CH_2)_8-CO-NH-(CH_3)_3-$	BPO_2–DAPS	25.5	3	6	5	5	4	5	5	8	5	7	4	5	5	7
$-(CH_2)_3-C_6H_4-C_6H_4-(CH_2)_3-$	BPO_2–APD	18§	Neg	4	4	4	2	4	4	6	3	5	3	5	3	5
$-(CH_2)_3-C_6H_4-S(=O)_2-C_6H_4-(CH_2)_3-$	BPO_2–DDS	20§	Neg	4	4	4	2	4	3	6	5	6	3	5	3	5
BPO_{15}–$PLL_{20}S$	BPL (multivalent)	—	6	10	8	14	8	14	7	13	7	12	6	9	7	9

* Duplicate tests; means reported; mean deviation ± 1 mm. Negative tests are 1–2-mm blebs. Reactions are BPO-specific as shown by complete or marked specific inhibition by the univalent haptens BPO-propylamine (administered with di- or multivalent haptens).

‡ Measured from Fisher-Hirshfelder molecular models.

§ The diphenyl and diphenylsulfone bridges are relatively rigid according to the molecular models.

outline surrounded by homogenous erythema; they were unusually pruritic. Reaction intensity was graded by average wheal diameter. This measurement increased with the concentration of the elicitor to describe a sigmoid dose-response curve. Thus, wheal diameter might increase from 3 to 15 mm as elicitor concentration was increased from 10^{-10} to 10^{-6} mole/liter. With careful injection technique, triplicate tests were in good agreement; average deviation from the mean wheal diameter was ± 1.0 mm. Two kinds of controls were done, hapten solutions injected into nonsensitive patients and TBS-EDTA diluent injected into BPO-hypersensitive patients. Both controls were negative, i.e., giving 1–2-mm blebs. BPO-hypersensitive patients utilized in this study were those who gave negative tests to the minor determinant mixture (8).

Passive transfer tests The Prausnitz-Kustner method was used: a 0.1 ml sample of sterilized serum was injected i.d. into the arm of nonsensitive recipients. After a latent period of 48 hr, test sites and nonsensitized control sites were tested by injection of 0.01 ml of hapten. Positive tests were typical wheal-and-flare reactions read at 15 min.

RESULTS

Effectiveness of bivalent haptens. Table II shows the results of testing seven patients simultaneously with the six differently sized bivalent haptens and a multivalent hapten. In general, the bivalent haptens were considerably less effective than was the multivalent hapten. However, in two patients (T. P. and C. A.), the bivalent (BPO_2-HMD) and multivalent haptens gave about equally intense reactions. Table III shows that sera from patients with strong skin reactivity to BPO_2-HMD were capable of passively sensitizing skin sites in nonsensitive recipients for reactivity to BPO_2-HMD, whereas sera from patients with poor skin reactivity to BPO_2-HMD failed to passively sensitize skin sites for reactivity to BPO_2-HMD, but did passively sensitize sites for reactivity to the multivalent hapten. Thus, this heterogeneity among individuals, with regard to the effectiveness of the bivalent relative to the multivalent hapten, is due to a factor relating to the patient's skin-sensitizing antibody, rather than to nonspecific factors related to the skin test site. This antibody factor is tentatively assumed to be the binding affinity, on the basis of analogy with other anaphylactic systems. Both in PCA in guinea pigs (3, 9) and in man passively sensitized with rabbit antibodies (10), bivalent haptens were found to elicit reactions with high-affinity antbodies, but not with low-affinity antibodies.

As to the effect of chain length separating the two haptenic groups in the divalent haptens, Table II shows the following: (*a*) BPO_2-PD with a maximum chain length of only 4.5 A (three methylene groups) was capable of eliciting specific W & F reaction. (*b*) BPO_2-HMD with a maximal separation of 8.5 A was a much more effective elicitor than was BPO_2-PD; and (*c*) further increase in the separation between the two BPO groups (maximal separations of 13, 17, and 26 A for the NMD, DMD, and DAPS derivatives) did not increase the effectiveness of the bivalent haptens. In fact, BPO_2-DMD and BPO-DAPS (with 17 and 26 A maximal separation appeared to be slightly less effective than was BPO_2-HMD (Table II, patients T. P. and C. A.).

As to the effect of rigidity, the two comparatively rigid bivalent haptens (Table II, BPO_2-

TABLE III

Relative Effectiveness of Bivalent Hapten in Active and Passive Wheal-and-Flare Reactions

Patient	Test reagents	Average wheal diameters*							
		Direct skin tests				Passive transfer tests‡			
		BPO_2-HMD		BPO_{15}-$PLL_{20}S$		BPO_2HMD		BPO_{15}-$PLL_{20}S$	
	Molar concentration..	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-8}	10^{-6}
		mm		*mm*		*mm*		*mm*	
J. W.		6	9	6	9	6	16	8	20
C. A.		5	8	7	8	7	9	10	12
F. X.		Neg	2	5	7	Neg	Neg	10	12
A. H.		Neg	3	5	9	Neg	Neg	6	10

* See footnote (*), Table II.

‡ In nonsensitive recipients, 0.1 ml of serum used per skin site, latent period, 48 hr.

TABLE IV

Comparative Effectiveness of Oligovalent and Polyvalent Haptens

Patients....	Average wheal diameters*								
	J. T.			E. M.			L. C.		
Hapten	Molar concentrations of haptens.... 10^{-11}	10^{-10}	10^{-9}	10^{-11}	10^{-10}	10^{-7}	10^{-8}	10^{-7}	10^{-6}
		mm			*mm*			*mm*	
PBO_2–HMD	Neg	2	4	Neg	2	4	Neg	2	3
BPO_3–Lys_3	Neg	4	5	Neg	2	4	Neg	2	3
BPO_4–Lys_4	Neg	4	5	Neg	3	5	Neg	3	4
BPO_6–Lys_7	Neg	4	5	Neg	4	7	Neg	6	6
BPO_{15}–$PLL_{20}S$	Neg	4	5	Neg	3	6	Neg	5	6
BPO_{135}–$PLL_{287}S$	Neg	4	5	Neg	3	6	Neg	4	6

* See footnote (*), Table II.

APD and BPO_2-DDS) were considerably less effective than were the flexible aliphatic haptens.

Effectiveness of oligovalent haptens. Table IV shows the results of testing simultaneously seven patients with bivalent, trivalent, quadrivalent, hexavalent, and the multivalent haptens. The 2-, 3- and 4-valent haptens gave relatively weak reactions. Maximally intense W & F reactions were obtained with the 6-valent hapten (BPO_6-Lys_7).[4] Effectiveness increased sharply between the 4- and 6-valent haptens. The 6-valent and the multivalent haptens (BPO_{15}-$PLL_{20}S$) were compared in a total of 40 patients with identical results shown in Table IV. Table IV also shows that threshold W & F reactions were elicited by equimolar concentrations of the bivalent, oligovalent, and multivalent haptens, which is consistent with previous results (1–3). Also, two patients showed smaller wheals to the large multivalent hapten (BPO_{135}-$PLL_{287}S$) than to the smaller haptens, particularly at the higher concentrations tested. This kind of observation has been made previously (1) and was shown to be due to relatively poor diffusion of the high molecular weight conjugates in skin (1).

[4] The following experiments were done to exclude the possibility that the high molecular weight impurity in the BPO_6-Lys_7 preparation might be responsible for its activity. BPO_6-Lys_7 was dialysed exhaustively against TBS, pH 8.2. Approximately 10% of the BPO concentration was not dialyzable, consistent with the chromatography findings (see Methods). The dialysate (low molecular weight fraction) was adjusted to 1×10^{-6} mole/liter concentration and compared with the undialyzed BPO_6-Lys_7 in seven BPO-sensitive patients. In all seven patients, the two preparations gave equally intense wheal-and-flare reactions.

Effectiveness of uni-, bi-, oligo-, and multivalent haptens in specific precipitation. In the preceding section the "valence" of the oligovalent haptens was accepted as the number of hapten groups per mole, i.e., a chemical valence. However, it was considered that the immunological valences of these haptens (i.e., the maximum number of antibody molecules bound per mole hapten), might be lower because of steric interference among the relatively large antibody molecules aggregated about these relatively small hapten molecules.

An approach to the measurement of the "immunological valences" of the oligovalent haptens was to determine the maximum antibody to hapten ratio in the specific precipitates of these oligovalent haptens with rabbit anti-BPO sera (in extreme antibody excess). Fig. 2 shows the precipitation of a pooled rabbit antiserum (BPO_{33}-BGG) by the oligovalent and multivalent haptens. This serum pool was from late bleedings of rabbits immunized with complete adjuvant, and accordingly should contain antibodies of relatively high binding affinity (11). The crucial finding was that for BPO_6-Lys_7 (the 6-valent hapten), the maximal antibody to hapten molecular ratio in the precipitate was 4 at extreme antibody excess, and toward the equivalence region the ratio fell to 3.

To study the effect of antibody binding affinity on this ratio, the serum pool was partially absorbed (see Methods) to remove preferentially the antibodies of higher binding affinities (11), and the quantitative precipitation experiments were repeated (Fig. 3). Here the maximal antibody to antigen molecular ratio in the precipitate for

Average wheal diameters*										
W. S.			J. C.			E. R.			J. M.	
10^{-9}	10^{-8}	10^{-7}	10^{-11}	10^{-9}	10^{-8}	10^{-11}	10^{-8}	10^{-7}	10^{-8}	10^{-7}
	mm			*mm*			*mm*			*mm*
Neg	2	4	3	3	5	Neg	5	5	3	4
Neg	2	4	3	4	5	Neg	5	5	3	4
Neg	3	6	3	5	5	Neg	6	6	6	6
Neg	6	10	3	6	9	Neg	6	8	7	9
Neg	5	9	3	6	8	Neg	6	8	7	9
Neg	4	6	3	4	5	Neg	6	7	7	9

BPO_6-Lys_7 was 3 in extreme antibody excess, falling to 2.5 toward the equivalence zone. Thus, for specific precipitation of rabbit antisera, the "immunological valence" of BPO_6-Lys_7 (chemically 6-valent) was at most 3–4, apparently depending, in part, on the binding affinity of the antibodies precipitated.

Of interest, Figs. 2 and 3 show that, in specific precipitation the bivalent haptens were ineffective, BPO_6-Lys_7 was only moderately effective,

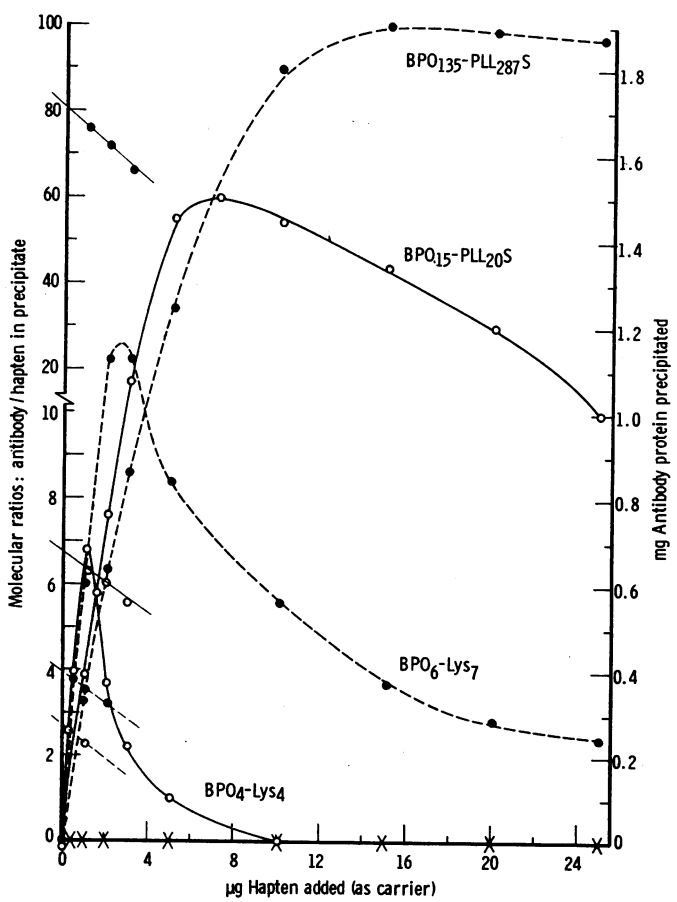

FIGURE 2 Specific precipitation of rabbit anti-BPO-serum (Pool 38) by divalent, oligovalent, and multivalent haptens. BPO_3-Lys_3 (not shown) precipitated antibody of 0.3 mg/ml of serum. X—X shows absence of precipitation by divalent haptens BPO_3-HMD, BPO_3-DMD, and BPO_3-DDS. Precipitation was completely and specifically inhibited by BPO-propylamine (univalent hapten).

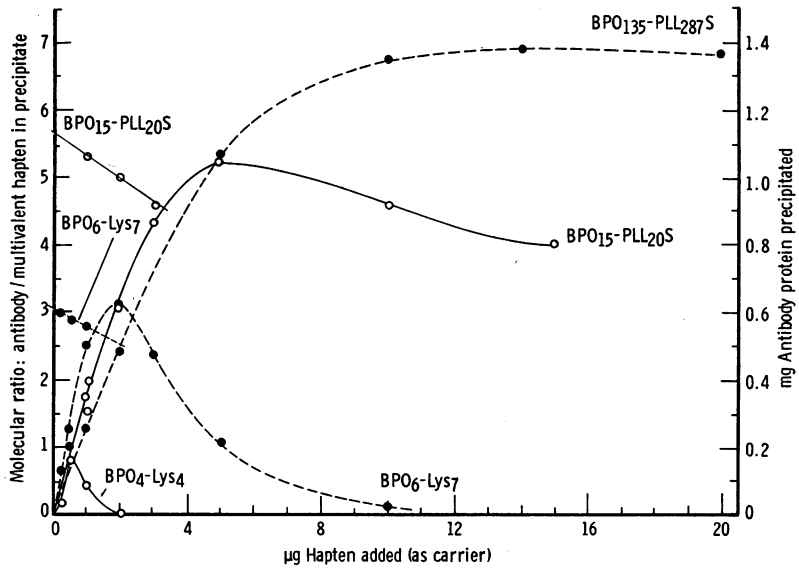

FIGURE 3 Specific precipitation of partially absorbed Pool 38 (rabbit anti-BPO serum) by divalent, oligovalent, and multivalent haptens. BPO_3-Lys_3 (not shown) precipitated a trace quantity of antibody. Divalent haptens (see legend, Fig. 2) did not precipitate serum.

and that the large multivalent hapten was required for maximal effectiveness. This contrasts sharply with effectiveness of these haptens in the W & F reaction. Also, by comparing the results in Figs. 2 and 3, it appears that the oligovalent haptens are relatively more effective in precipitation of antibodies of the higher affinities.

Effect of extent of conjugation of multivalent haptens on their effectiveness in specific precipitation and W & F. Fig. 4 shows the quantitative precipitation of anti-BPO sera by the multivalent BPO-$PLL_{535}S$ haptens with 7, 12, 24, 52, and 139 BPO groups per mole (averages). Maximal precipitation was acheived with BPO_{52}-$PLL_{535}S$,

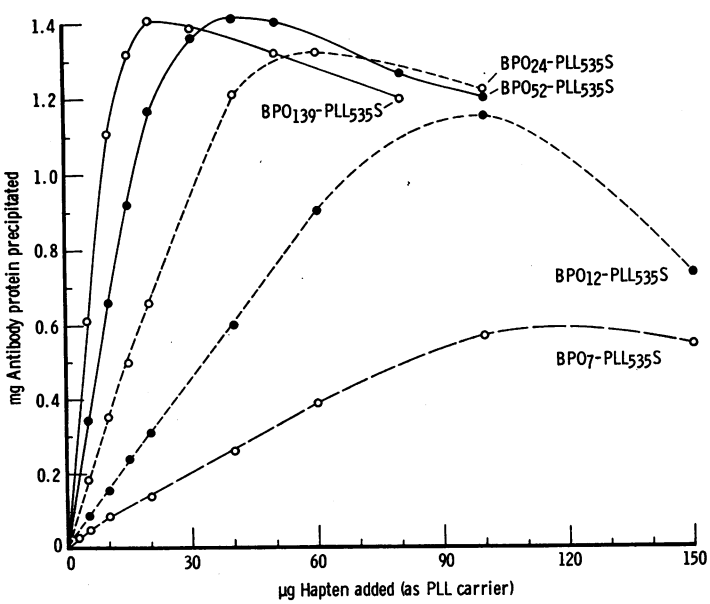

FIGURE 4 Specific precipitation of rabbit anti-BPO serum (Pool 39B) by multivalent BPO-$PLL_{535}S$ conjugates of varying extents of conjugation, BPO_7-$PLL_{535}S$; BPO_{12}-$PLL_{535}S$; BPO_{24}-$PLL_{535}S$; BPO_{52}-$PLL_{535}S$; BPO_{139}-$PLL_{535}S$. Precipitation was completely and specifically inhibited by BPO-propylamine (univalent hapten).

TABLE V

Effect of Extent of Conjugation of a Multivalent Hapten (BPO–PLL$_{535}$S) on Elicitation of Wheal-and Flare Reaction

Conjugate	Average wheal diameters* Patients.... F. R.		L. L.		S. S.	
	Conjugate concentrations (*mole/liter*).... 10^{-8}	10^{-7}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
	mm		*mm*		*mm*	
BPO_7–$PLL_{535}S$	Tr.	4	Neg	3	Neg	Tr.
BPO_{12}–$PLL_{535}S$	3	5	Tr.	5	3	5
BPO_{24}–$PLL_{535}S$	5	7	6	8	5	7
BPO_{52}–$PLL_{535}S$	7	10	8	9	7	8
BPO_{135}–$PLL_{535}S$	7	10	8	9	7	8

* See footnote (*), Table II.

but BPO_7-$PLL_{535}S$ was capable of precipitating 42% and BPO_{12}-$PLL_{535}S$ was capable of precipitation of 81% of the total precipitable antibody. By contrast, the BPO_{12} conjugate elicited only weak W & F reactions and BPO_7-$PLL_{535}S$ gave only trace reactions (Table V). In BPO_{12}-$PLL_{535}S$, the haptenic groups would be expected to be generally widely spaced, assuming random coupling. That the BPO groups are relatively widely spaced in the lightly coupled conjugates is indicated by the data in Fig. 5. For these conjugates, the maximum antibody-hapten molecular ratios in precipitates in extreme antibody excess were equal to average numbers of BPO groups per molecule. Where the haptenic groups were relatively close together, i.e. in heavily coupled conjugates, the maximum antibody-hapten ratio in the precipitate was considerably lower than the average number of BPO groups per mole conjugate.

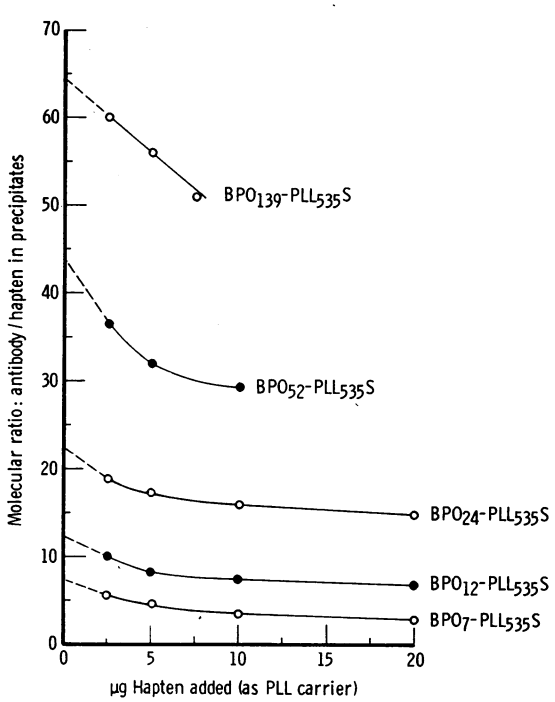

FIGURE 5 Antibody-hapten ratios in precipitate in antibody excess zone from data in Fig. 4.

DISCUSSION

The foregoing results suggest that trivalent haptens whose haptenic groups are separated by optimal distances are generally required for maximally effective elicitation of the specific wheal-and-flare reaction (W & F). Divalent haptens, although variably effective, were generally not maximally effective elicitors of W & F. The smallest oligovalent eliciting maximaly intense W & F was BPO_6-Lys_7. Although the latter is chemically a 6-valent hapten, it appears to be trivalent with regard to elicitation of W & F, i.e., capable of binding three antibody molecules per molecule hapten. This was not determined directly but inferred from the following considerations. In specific precipitation of late immunization rabbit anti-BPO sera, BPO_6-Lys_7 bound a maximum of three or four antibody molecules per mole hapten in extreme antibody excess, i.e., its "immunological valence" was 3 or 4. The "immunological valence" of BPO_6-Lys_7 was 4 in precipitation of high-affinity antibodies, and 3 in precipitation of low-affinity antibodies. Considering that hapten-antibody binding takes place in three dimensions in precipitation, where it is likely to take place in two dimensions in the elicitation of W & F, its valence in the latter situation would be more likely to be 3. In support of this inference, a scale model (Fig. 6) can be drawn showing BPO_6-Lys_7 to be sterically capable of coplanar binding of three antibody molecules (assuming a small diameter of 40 A for antibody molecules and a close approximation of the antibodies). According to this model, BPO_6-Lys_7 is sterically incapable of coplanar binding of four antibody molecules.

An important question is whether BPO_6-Lys_7 exists aggregated in solution. It appears to us unlikely that BPO_6-Lys_7 exists in aggregated form in the concentrations used for elicitation of

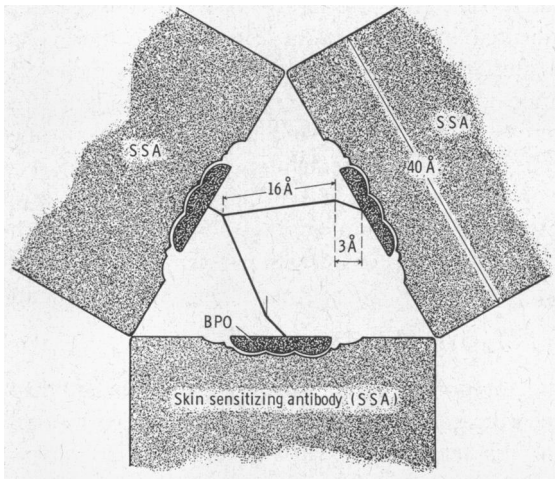

FIGURE 6 Scale model of coplanar binding of skin-sensitizing antibodies (*SSA*) by BPO_6-Lys_7. Only three of the six BPO groups are drawn in. Hapten lengths were measured from Fisher-Hirshfelder scale molecular models. The model assumes a small diameter of 40 A for *SSA* and a close approximation of the antibody molecules.

W & F (10^{-6} to 10^{-10} mole/liter) although this was not definitely proven. However, the divalent BPO haptens did not appear to aggregate appreciably since they did not precipitate with rabbit anti-BPO sera, and there was a regular increase in specific precipitability of the oligovalent haptens from BPO_3-Lys_3 to BPO_4-Lys_4, to BPO_6-Lys_7, to BPO_{15}-Lys_{20} to reach maximum precipitability with BPO_{135}-Lys_{287}.

The requirement for optimal separation of the haptenic groups is indicated by the following data. BPO_3-Lys_3 and BPO_4-Lys_4, in which haptenic groups are closely spaced, were generally not maximally effective elicitors. These molecules are too small to effect coplanar binding of three antibody molecules when tested in the scale model in Fig. 6. On the other hand, BPO_7-$PLL_{535}S$ and BPO_{12}-$PLL_{535}S$ which can bind three and seven antibody molecules per mole hapten, but in which the BPO groups appear to be statistically widely separated, were also not maximally effective elicitors. Further, the bivalent haptens in which the haptenic groups were separated by an aliphatic chain of 9 or 13 A were generally more effective elicitors than were the bivalent haptens with aliphatic chains of 17 to 26 A, or a bivalent hapten with a separation of 3.5 A.

The apparent, general requirement for trivalency with optimal separation of haptenic groups may reflect a requirement for the formation of a comparatively high-energy complex made up of two or three membrane-fixed skin-sensitizing antibodies closely bound together by the hapten as the initiator of W & F.[5] The comparative ineffectiveness of the bivalent haptens as elicitors of W & F, particularly with antibodies of low-binding affinities, may be tentatively ascribed to the relatively high statistical probability of one of the hapten-antibody bonds of the $H\text{-}Ab_2$ complex breaking which thus disrupts the complex. Disruption of the $H\text{-}Ab_3$ complex formed from the trivalent hapten would require simultaneous disruption of two hapten-antibody bonds, an event which carries a considerably lower statistical probability. Stated in energetic terms, the $H\text{-}Ab_3$ complex would have a considerably higher bond energy than would the $H\text{-}Ab_2$ complex, due to summation of the individual H-Ab bond energies and possibly, also because of an additional entropy contribution.

The actual site of antigen-antibody reaction leading to specific wheal formation is not clearly known, although it is likely to take place on the surface of mast cells in skin. The precise molecular mechanisms involved in the initiation process is also not clearly known. However, it has been hypothesized that the initiation of anaphylactic reactions by complexes may involve the induction of toxic allosteric changes in the anaphylactic antibody molecules by binding the antigen (12), or that such complexes may activate a lytic enzyme (13–15). We should like to propose a third possibility, that, the rigidification of a portion of an actively motile cell membrane (e.g., of a mast cell) by the closely bridged complex described herein may stimulate mast cell degranulation by a simple mechanical process.

ACKNOWLEDGMENTS

We are pleased to acknowledge the excellent technical assistance of Miss Vera Levytska in this study.

This study was supported by grant U-1297 from the Health Research Council of the City of New York.

[5] The possibility that one skin-sensitizing antibody (SSA) molecule may bind the two or three haptenic groups of the bivalent or oligovalent hapten cannot be excluded by this data, but appears unlikely. To form such a complex would require that the specific binding sites of the SSA molecule would have be brought to within 5–16 A of each other. This would appear unlikely for the SSA molecule.

REFERENCES

1. Levine, B. B., and M. J. Fellner. 1965. The nature of immune complexes initiating allergic wheal-and-flare reactions. *J. Allergy.* **36:** 342.
2. Levine, B. B. 1965. The nature of the antigen-antibody complexes which initiate anaphylactic reactions. II. The effect of molecular size on the abilities of homologous multivalent benzylpenicilloyl haptens to evoke PCA and passive arthus reactions in the guinea pig. *J. Immunol.* **94:** 121.
3. Levine, B. B. 1965. The nature of the antigen-antibody complexes which initiate anaphylactic reactions. I. A quantitative comparison of the abilities of nontoxic univalent, toxic univalent, divalent and multivalent benzylpenicilloyl haptens to evoke passive cutaneous anaphylaxis in the guinea pig. *J. Immunol.* **94:** 111.
4. Waley, S. G., and J. Watson. 1953. The action of trypsin on polylysine. *Biochem. J.* **55:** 328.
5. Stewart, J. W., and M. A. Stahmann. 1962. The chromatography of polylysine. *J. Chromatog.* **9:** 233.
6. Levine, B. B. 1962 (α-D-penicilloyl) amines as univalent hapten inhibitors of antibody dependent allergic reactions to penicillin. *J. Med. Pharm. Chem.* **5:** 1025.
7. Levine, B. B. 1964. The preparation of penicilloyl-polylysines, skin test reagents for the clinical evaluation of penicillin hypersensitivity. *J. Med. Chem.* **7:** 675.
8. Voss, H. E., A. P. Redmond, and B. B. Levine. 1966. Clinical detection of the potential allergic reactor to penicillin by immunologic tests. *J. Am. Med. Assoc.* **196:** 679.
9. Hurlimann, J., and Z Ovary. 1965. Relationship between affinity of anti-dinitrophenyl antibodies and their biologic activities. *J. Immunol.* **95:** 765.
10. Parker, C. W., M. Kern, and H. N. Eisen. 1962. Polyfunctional dinitrophenyl-haptens for elicitation of immediate type allergic skin responses. *J. Exptl. Med.* **115:** 789.
11. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochem.* **3:** 996.
12. Ishizaka, K., and D. H. Campbell. 1959. Biological activity of soluble antigen-antibody complexes. V. Change of optical rotation by the formation of skin reactive complexes. *J. Immunol.* **83:** 318.
13. Austen, K. F., and W. E. Brocklehurst. 1961. Anaphylaxis in chopped guinea pig lung. I. Effect of peptidase substrates and inhibitors. *J. Exptl. Med.* **113:** 521.
14. Mongar, J. L., and H. O. Schild. 1962. Cellular mechanisms in anaphylaxis. *Physiol. Rev.* **42:** 226.
15. Perera, B. A. V., and J. L. Mongar. 1963. The role in anaphylaxis of a chymotrypsin-like enzyme in rat mast cells. *Immunology.* **6:** 478.