Immunology, 1964, **7**, 542.

Studies on the Immunological Mechanisms of Penicillin Allergy

II. ANTIGENIC SPECIFICITIES OF ALLERGIC WHEAL-AND-FLARE SKIN RESPONSES IN PATIENTS WITH HISTORIES OF PENICILLIN ALLERGY*

B. B. LEVINE AND VERA H. PRICE

Departments of Medicine and Dermatology, New York University School of Medicine

(*Received* 30*th October* 1963)

Summary. Forty-one patients with acceptable past histories of allergic reactions to benzylpenicillin (PG), eleven patients with questionable histories to PG and thirty patients without past histories of allergic reactions to PG were skin tested with various multivalent haptenic conjugates and with simple chemicals derived from PG in order to determine the antigenic specificities of penicillin hypersensitivity of the wheal-and-flare type. The benzylpenicilloyl (BPO) group was found to be the major haptenic determinant of wheal-and-flare type PG hypersensitivity. Twenty-nine per cent of patients with acceptable histories of PG allergy and 3 per cent of patients without histories of PG allergy gave positive wheal-and-flare reactions to multivalent BPO-conjugates. Three patients who had unusual clinical forms of PG allergic reactions demonstrated patterns of wheal-and-flare reactivity indicating D-penicillamine or D-benzylpenamaldic acid disulphide haptenic specificity. No unequivocal wheal-and-flare reactivity specific for the benzylpenicillenic acid haptenic group was observed in this study. Data were obtained which indicate that BPO-specific wheal-and-flare skin reactivity demonstrates specificity for the entire large BPO haptenic group, and also for structural areas of the immunizing autologous hapten-carrier protein (i.e. carrier specificity).

INTRODUCTION

Previous studies have demonstrated that the benzylpenicilloyl group is a major haptenic determinant of human penicillin hypersensitivity of the wheal-and-flare type (Levine and Ovary, 1961; Parker, Shapiro, Kern and Eisen, 1962). In addition, Parker *et al.* (1962) have reported that the benzylpenicillenic acid disulphide group may also be a haptenic determinant of human penicillin hypersensitivity of the wheal-and-flare type.

The present paper describes the results of further studies on the antigenic specificities of benzylpenicillin (PG) hypersensitivity of the wheal-and-flare (W&F) type in human

* The abbreviations used in this paper are: PG = benzylpenicillin (penicillin G); KPG = potassium PG; BPO = benzylpenicilloyl haptenic group; α-diastereoisomeric BPO group; BPE = benzylpenicillenic acid haptenic group; PSH = D-penicillamine haptenic group; BPO–EACA = BPO–ε-aminocaproate (univalent hapten); PLL = poly-L-lysine; HSA = human serum albumin; HGG = human γ-globulin; α-BPO_{72}–$PLL_{402}S$ = multivalent α-BPO conjugate of PLL whose free amino groups are succinylated. Subscripts are average number of residues per molecule of conjugate; BPE_8–acHSA = BPE conjugate of acetylated HSA; TBS = Tris (0·02 M) buffered saline, pH 8·0; W&F = wheal-and-flare reaction.

beings. Groups of patients with and without past histories of allergic reactions to penicillin were skin tested with the various multivalent hapten conjugates derived from PG whose preparations are described in Part I (Levine, 1964), and with several low molecular weight chemicals related to PG. Specificities of the W&F reactions were confirmed by specific inhibition of W&F reactions with univalent haptens.

The results obtained confirm that the benzylpenicilloyl (BPO) group is the major haptenic determinant of W&F allergic skin reactivity in human beings hypersensitive to PG. Also, three patients were observed who demonstrated patterns of W&F reactivity indicating hypersensitivity to the D-penicillamine and/or the D-benzylpenamaldic acid disulphide haptenic determinant. In contrast to the results of Parker *et al.* (1962), unequivocal W&F reactivity specific for the benzylpenicillenic acid haptenic group was not observed. Finally, data were obtained which may be best interpreted as indicating that immunological specificity of anti-BPO-antibodies mediating the W&F reaction is directed also toward structural configurations of the autologous protein carrier which induced the immune response (i.e. carrier specificity).

MATERIALS AND METHODS

Skin Test Materials

Crystalline KPG was generously supplied by Dr. J. Doyle of Bristol Laboratories, Syracuse, New York. Monsodium benzylpenicilloate.$\frac{1}{2}$.H_2O was prepared and crystallized twice from water-acetone as described previously (Levine, 1960a), m.p. 154–155°. It was found to be free of PG by bioassay (Grove and Randall, 1955). D-Penicillamine.HCl.$\frac{1}{2}$.H_2O was purchased from California Corporation, Los Angeles. Its nitroprusside test was strongly positive. Crystalline α-BPO–EACA and the equilibrium diastereoisomeric mixture of BPO–EACA were the preparations described in Part I (Levine, 1964). Multivalent BPO–protein conjugates, succinylated BPO–PPL, BPE_8–acHSA and PSH_{10}–HSA conjugates were the preparations described in Part I. In addition, a conjugate containing a diastereoisomeric mixture of BPO groups, DM-BPO_{58}–$PLL_{402}S$, was prepared by incubating a solution of α-BPO_{72}–$PLL_{402}S$ in phosphate buffered saline (PBS), pH 5·5 under N_2 at 56° for 6 hours. Optical rotation measurement done on the α-BPO_{72}–$PLL_{402}S$ solution and corrected for the contribution of the $PLL_{402}S$ carrier indicated an α for 1×10^{-2} M BPO-amine of +0·96° (2 dm. tube, sodium light, 0·3 per cent PLL base in PBS, pH 8) which is in excellent agreement with the molar specific rotation of crystalline α-diastereoisomeric BPO-amines (Levine, 1962). The optical rotation of 1×10^{-2} M BPO-amine was obtained in the same way for DM-BPO_{58}–$PLL_{402}S$ and indicated an α of +0·65° which is somewhat higher than the molar specific rotations of sterically equilibrated BPO-amines (Levine, 1962). These α-BPO and DM-BPO conjugates contained, respectively, averages of 0·5 and 3·0 benzylpenamaldoyl groups per mole (formed by rearrangement of BPO). Also, considering the fall of the number of BPO groups per mole conjugate resulting from mutarotation of α-BPO_{72}–$PLL_{402}S$, some degradation of BPO groups to benzylpenaldoyl groups or oxidation products of benzylpenaldoyl groups might have occurred during mutarotation.

Skin test reagents were analysed quantitatively as described in Part I. They were diluted in TBS, sterilized by filtration through Swinney filters using Millipore HA filter membranes, and stored at 4° in rubber capped allergy vials. Conjugates were stable in this form during a 6-month period of observation. Conjugate concentrations are expressed

in mg./ml. of the carrier protein or polypeptide. Simple chemical reagents were dissolved in TBS immediately before use, although KPG solutions could be stored at 4° for at least 1 month without detectable loss in ability to provoke positive W&F reactions.

Test Subjects

Subjects were patients on the wards and in the clinics of the Third and Fourth Medical and Dermatology Services (New York University) of Bellevue Hospital. Some of the patients listed in Tables 3 and 5 were tested at the Roosevelt Hospital, Institute of Allergy, New York City. Subjects were of both sexes, and between 18 and 70 years of age. They were generally recovering from either traumatic or infectious diseases, and were in good general condition. All patients had received penicillin one or more times in the past either orally or parenterally. Presumably most of the penicillin administered to these patients had been benzylpenicillin, although no documentation of this was generally available.

A history of penicillin allergy was considered acceptable if the patient on repeated questioning gave a consistent history of one or more of the following symptoms occurring within 1 week after penicillin administration: (1) rash, either urticarial, diffuse erythematous or maculo-papular; (2) serum sickness-like reactions; (3) angioedema; (4) symptoms of generalized vasodilitation such as flushing, lightheadedness, loss of consciousness, or acute asthma occurring within 2 hours after penicillin administration. Three of the forty-one patients judged to have acceptable histories claimed to have had the onset of urticarial reactions 2 weeks after penicillin therapy was stopped (including one reactor, J.L., Table 3). In many of these cases medical documentation was not available. A history of penicillin allergy was judged to be questionable for the following reasons: (1) The history was vague and was not consistent on repeated questioning (four patients). (2) The patients had localized inflammatory reactions, e.g. swelling of mouth and jaw after penicillin intramuscularly and a local dental anaesthetic, or a 'rash only on the neck' (three patients). (3) A syndrome which was more likely not due to penicillin allergy, e.g. arthralgia without skin eruption following PG treatment of a streptococcal pharyngitis (three patients) or non-thrombocytopenic purpura alone (one patient). In the majority of cases, patients had been treated also with at least one other drug (most commonly, an aspirin preparation) before the occurrence of the allergic reaction. All cases were interviewed by both authors, and classification was based on joint clinical impression prior to skin testing.

Skin Testing Methods

Disposable 0·5 ml. syringes and $\frac{3}{8}$ in. 26-gauge intradermal bevel needles were used, once only (Hypak B-D Co., Rutherford, New Jersey). Test volumes sufficient to raise a just detectable wheal (estimated at approximately 0·005 ml.) were generally used to test for W&F reactivity. Up to ten tests were placed into the postero-lateral skin surface of each arm from about 7 cm. below the acromial process to 7 cm. above the elbow (two rows of five tests). These skin sites were found to be generally equal in reactivity. Some variability in wheal responses were found when several tests with the same reagent were done simultaneously. These variations in average wheal diameters (e.g. in a typical subject the diameters of four wheals might range between 8 and 10 mm.) were not related to the skin site used and were presumably due to slight variations in injection technique. Accordingly, skin tests were done in duplicate where possible. Skin sites were examined in

15 minutes, and the average diameters of the wheals and surrounding erythema were recorded. Negative reactions were non-pruritic blebs of 1–3 mm. diameter, and were of smooth and indistinct outline. They were generally without surrounding erythema, but in approximately 10 per cent of the cases they were surrounded with up to 10 mm. of stippled erythema. Positive reactions were wheals of sharp and irregular outline with surrounding homogeneous intense erythema. Positive reactions were generally, but not always, pruritic. Positive W&F reactions appeared within 3–5 minutes and were at their maximum intensities within 10–12 minutes. W&F reaction intensities were based on average wheal diameters: 1–3 mm., negative; 4–6 mm., equivocal; 7–9 mm., weak; 11–17 mm., moderate; over 17 mm. (usually with pseudopods), strong. In about half the subjects, test sites were examined in 24 hours for delayed skin reactions.

RESULTS

PRIMARY IRRITANCY OF SKIN TEST REAGENTS IN NON-ALLERGIC PATIENTS

Reagents were injected in a 0·05 ml. volume and volume sufficient to raise a just detectable wheal (0·002–0·005 ml.) in order to determine the effect of volume on non-specific irritancy of the reagents. The larger intradermal test volume (0·05 ml.) was appreciably more irritating than was the small volume necessary to raise a just detectable wheal. Two of sixteen patients without histories of penicillin allergy reacted to the 0·05 ml. test volume of diluent with 11–12 mm. wheals and surrounding diffuse erythema. The other fourteen patients gave 8–10 mm. wheals without erythema, which represents slight spreading of the intradermal fluid deposit during the 15 minutes of observation. All sixteen patients reacted to the minimal volume with 2–3 mm. wheals and only one patient showed slight surrounding stippled erythema. The mild inflammatory reactions appeared consistently on retesting the patients who gave non-specific reactions. Similar non-specific reactions were obtained in the two non-specific reactors with freshly prepared phosphate buffered saline solutions at pH 7·0, 7·5 and 8·0, with commercial sterile physiological saline from freshly opened bottles, and with these materials at 4°, 25° and at 37°. These reactions were not associated with pruritus in contrast to the majority of specific allergic wheals which were associated with pruritus. Similar non-specific W&F reactions have been observed by MacLeod in 10 per cent of normal patients injected intradermally with physiological saline (C. M. MacLeod, personal communication). Although the mechanism of this inflammatory response is unknown, it appears to be related to the trauma of the intradermal injection.

With the exception of BPO_{22}–HGG, the test reagents were non-irritating, giving responses indistinguishable from those of the diluent. BPO_{22}–HGG exhibited considerably greater primary irritancy when injected in the 0·05 ml. volume than when injected in the smaller volume. Accordingly all skin test studies were made using the just detectable wheal injection.

BENZYLPENICILLOYL (BPO) HAPTENIC SPECIFICITY OF ALLERGIC WHEAL-AND-FLARE SKIN REACTIVITY

Forty-one patients with acceptable past histories of allergic reactions following treatment with penicillin, eleven patients with questionable histories, and thirty patients without past histories of allergic reactions to PG, were skin tested with PG and with

various multivalent conjugates of the BPO haptenic group, BPE group, and PSH group. All of these patients had received penicillin therapy in the past. As shown in Tables 1 and 2, 29 per cent of patients with acceptable histories of penicillin allergy, none of the patients with questionable histories, and one patient (3 per cent) without a history of penicillin allergy gave unequivocal W&F reactions to the BPO conjugates. This last patient had received penicillin many times in the past, and may accordingly have become hypersensitive to the BPO haptenic determinant. Further studies could not be carried out on this patient. Specific inhibition of the W&F reactions with univalent BPO haptens (BPO–EACA) was carried out in eight patients. Table 3 shows that complete or almost complete inhibition was observed in six patients and marked inhibition was observed in the other two patients. These latter two patients gave the most intense W&F reactions to the multivalent conjugates alone, and complete inhibition might have been achieved in these patients too if lower concentrations of BPO_{53}–$PLL_{402}S$ had been used. These specific (see footnote to Table 3) inhibition data demonstrate that the BPO group is the haptenic group responsible for W&F reactions provoked by BPO_{53}–$PLL_{402}S$. Similarly, virtually complete inhibition of W&F reactions provoked by BPO_{22}–HGG at 50 μg./ml. and by α-BPO_{26}–$PLL_{402}S$ at 10 μg./ml. was achieved by BPO–EACA at 1×10^{-3} M concentration in two patients (not tabulated).

TABLE 1

FREQUENCY OF WHEAL-AND-FLARE REACTIONS TO MULTIVALENT BENZYLPENICILLOYL (BPO) CONJUGATES*

History of penicillin allergic reaction	*No. of patients tested*	*Positive*			*Equivocal*	*Negative*
		Strong	*Moderate*	*Weak*		
None	30	1(3%)	0	0	0	29(97%)
Questionable	11	0	0	0	1(9%)	10(91%)
Acceptable	41	3(7·3%)	5(12%)	4(10%)	1(2·5%)	28(68%)

* See Material and Methods section for classification of patients and descriptions of reactions.

D-PENICILLAMINE (PSH) HAPTENIC SPECIFICITY OF ALLERGIC WHEAL-AND-FLARE SKIN REACTIVITY

Two patients gave W&F reactions to a multivalent PSH conjugate, PSH_{10}–HSA. Patient C.H. (Table 2) had a personal history of multiple atopic allergic diseases and of a past immediate systemic allergic reaction to penicillin. He gave intense W&F reactions to multivalent BPO conjugates, and moderately intense reactions to KPG and to BPO–EACA. Patient M.B. did not have a personal history of atopic allergic disease. She was skin tested 1 week after a late urticarial allergic reaction to PG and was found to react to BPO_{22}–HGG (Table 2). She was not tested with PSH_{10}–HSA or with KPG at this time. During the next 4 months she experienced recurrent episodes of diffuse urticaria. Four months after the first skin test was performed, repeat skin tests showed moderately intense W&F reactivity to KPG and to crystalline sodium benzylpenicilloate, weaker W&F reactions to PSH_{10}–HSA and negative reactions to the multivalent BPO and BPE conjugates (Table 4). At this time the patient stopped ingesting her usual large quantities of milk (which may contain PG and degradation products of PG, see below) with a marked

TABLE 2

ALLERGIC WHEAL-AND-FLARE REACTIONS TOWARD KPG AND MULTIVALENT CONJUGATES DERIVED FROM PENICILLIN

Patient, age (yr.) and sex*	*Personal allergic history*	*History of penicillin allergy*	*Time elapsed since allergic reaction*	*Test material and concentration in mg. per ml. of carrier†*												*Classification of W&F reactions‡*
				Diluent (PBS or TBS)	*KPG (10,000 units/ml.)*	*BPO_6–$PLL_{20}S$* 0·2	2·0	*BPO_{170}–$PLL_{750}S$* 0·2	2·0	*BPO_{22}–HGG* 0·2	2·0	*BPO_{13}–HSA* 0·2	2·0	*BPE_8–acHSA* 0·15	1·5	*PSH_{10}–HSA* 0·4	4·0	
1 R.U. 44 F	None	None	—	3;0	3;0	—	22P;60	—	—	—	—	—	—	—	—	—	—	Strong
2 B.S. 20 F	Chronic urticaria	Questionable	16 yr.	3;0	3;0	—	6;10	—	—	—	—	—	—	—	3;0	—	3;0	Equivocal
3 M.C. 20 F	Perennial asthma	Late urticaria	1 wk.	(3;4)	(4;6)	4;7	(6;20)	3;4	(6;17)	(4;7)	4;0	(5;7)	7;0	5;8	5;0	4;7	5;7	Equivocal
4 G.C. 68 M	None	Late urticaria	1 wk.	3;0	(5;15)	(5;30)	(7;20)	—	—	3;0	6;11	4;0	7;15	3;0	3;0	3;0	3;0	Weak
5 N.C. 31 F	None	Late urticaria	6 wk.	2;0	3;0	9;30	—	7;30	7;30	5;10	7;12	5;15	5;30	2;0	2;0	2;0	2;0	Weak
6 M.Q. 46 M	None	Late urticaria	10 mth.	(4;10)	(4;10)	—	—	—	10;12	8;15	10;25	—	7;15	—	3;0	—	4;0	Weak
7 J.L. 40 M	None	Late urticaria	6 wk.	3;0	2;0	9;25	—	7;15	—	5;0	7;15	4;8	—	2;0	2;0	2;0	2;0	Weak
8 M.B. 24 F	None	Late urticaria	3 wk.	—	—	—	—	—	—	—	11;35	—	—	—	4;0	—	—	Moderate
9 G.Mc. 47 M	None	Diffuse erythema	10 mth.	3;0	(5;15)	16;35	—	11;40	—	11;35	13;35	8;35	—	2;0	2;0	2;0	2;0	Moderate
10 G.M. 57 M	None	Serum sickness	4 wk.	2;0	2;0	15;35	18;35	13;30	20;40	10;30	10;30	7;18	—	2;0	2;0	3;0	3;0	Moderate
11 N.S. 53 M	None	Late urticaria	5 yr.	3;0	—	—	—	13;30	—	15;40	—	—	—	3;0	—	3;0	—	Moderate
12 F.G. 41 F	None	Erythema nodosum	3 wk.	3;0	3;0	10;45	0;0	16;45	—	6;30	—	7;30	—	3;0	3;0	3;0	3;0	Moderate
13 C.H. 23 M	Seasonal rhinitis and asthma, Insect sting Immed. urticaria	Immed. urticaria	5 mth.	3;0	7;15	22P;60	—	—		14;30	—	14;40	—	5;0	7;10	5;15	10;10	Strong
14 C.W. 50 F	Chronic urticaria	Late urticaria	1 yr.	3;0	3;0	22P;50	—	—		—	—	—	—	—	3;0	—	3;0	Strong
15 V.W. 39 F	None	Immed. urticara	5 yr.	3;0	3;0	—	—	—		18P;50	—	—	—	4;0	4;0	4;0	4;0	Strong

* Fifteen reactors from forty-one patients with acceptable histories of penicillin allergy, eleven patients with questionable histories and thirty patients with no histories of penicillin allergy. The other sixty-seven patients gave negative reactions to KPG and the multivalent conjugates.

† W&F reactions at 15 minutes were expressed as average diameter of wheal followed by average diameter of surrounding erythema in mm. Erythema was homogenous and strong except those in parentheses which indicates faint and stippled erythema. P = pseudopods. Test materials were non-irritant except for BPO_{22}–HGG (Table 1). See footnote on page 542 for abbreviations.

‡ Reaction intensities are based on average wheal diameter: 1–3 mm., negative; 4–6 mm., equivocal; 7–9 mm., weak; 10–17 mm., moderate; over 17 mm. (usually with pseudopods, strong).

TABLE 3

SPECIFIC INHIBITION OF WHEAL-AND-FLARE RESPONSES IN THE BPO SYSTEM BY UNIVALENT HAPTENS*

Subject†	*Test material*‡				
	BPO_{53}–$PLL_{402}S$	*BPO_{53}–$PLL_{402}S$ + α-BPO–EACA*	*BPO_{53}–$PLL_{402}S$ + BPO–EACA (DM)*	*BPO–EACA + α-DM*	*Diluent TBS*
V.J.	11;30	4;0	–	3;0	1;0
T.F.	9;20	2;0	–	2;0	1;0
S.A.	11;35	6;10	5;TR	3;0	2;0
E.M.	12;30	3;TR	2;0	2;0	1;0
E.S.	18P;40	10;20	8;20	2;0	2;0
J.B.	8;30	2;0	2;0	1;0	1;0
J.N.	15P;40	7;10	7;10	2;0	2;0
I.P.	8;20	4;TR	2;0	2;0	2;0

* Inhibition was specific as BPO–EACA preparations did not inhibit moderate W&F reactions provoked by 10PNU/ml. ragweed extract in three ragweed-sensitive patients. Skin tests done in duplicate; mean diameters tabulated; mean deviation, ±1 mm. (for wheal). See footnote to Table 2 for expression of W&F responses. P = pseudopods.

† Patients with histories of penicillin allergy.

‡ Concentrations: BPO_{53}–$PLL_{402}S$, 10 μg./ml. (of PLL); α-BPO–EACA and BPO–EACA (DM), $1{\cdot}0 \times 10^{-3}$ moles/l. See footnote on page 542 for abbreviations.

TABLE 4

WHEAL-AND-FLARE REACTIONS OBTAINED IN PATIENT M.B.*

Concentration (mg./ml.)	*Multivalent conjugates*†				
	BPO_{22}–HGG	*BPO_{13}–HSA*	*BPO_{170}–$PLL_{750}S$*	*BPE_8–acHSA*	*PSH_{10}–HSA*
2·0	2;0	2;0	2;0	2;0	6;18
0·2	2;0	2;0	2;0	2;0	6;18
0·02	2;0	2;0	2;0	2;0	4;8
0·002	2;0	2;0	2;0	2;0	2;0

Concentration (moles/l.)	*Simple chemicals*†		
	Potassium penicillin G	*Sodium benzylpenicilloate*	D-*Penicillamine*
$1{\cdot}8 \times 10^{-2}$‡	10;40	9;35	2;0
$1{\cdot}8 \times 10^{-3}$	8;30	6;20	2;0
$1{\cdot}8 \times 10^{-4}$	5;10	–	2;0
$1{\cdot}8 \times 10^{-5}$	–	–	2;0

* Tests done 4 months after allergic reaction. Tests done 4 days after reaction are listed in Table 2, and tests done 14 months after reaction were entirely negative. See footnote to Table 2 for expression of W&F reactions. –, not done.

† Diluent TBS gave negative reaction (2;0) Test materials were non-irritant.

‡ Approximately 10,000 units/ml. of KPG.

decrease in the frequency and intensity of the urticarial episodes. Repeat skin tests performed 15 months after the initial allergic reaction were negative to all reagents listed in Table 4 and also to α-BPO_{58}–$PLL_{402}S$.

A third patient (E.N.) had been observed previously* who had experienced a 6-month course of recurrent urticara following a late urticarial reaction to PG. During this time, he experienced an episode of anaphylactic shock with diffuse urticaria, whose etiology

* At the Allergy Clinic, the Mount Sinai Hospital, New York City.

could not be established (but was presumably due to an unknown contact with penicillin, e.g. in milk). On skin testing, E.N. gave moderately intense W&F reactions to PG and to crystalline sodium benzylpenicilloate (2×10^{-3} molar concentrations). These reactions were specifically and almost completely inhibited by 2×10^{-2} molar D-penicillamine-cysteine mixed disulphide prepared by the method of Tabachnick, Eisen and Levine, (1954). Skin tests with multivalent conjugates were not done. These patterns of W&F reactivity indicate D-penicillamine haptenic specificity and possibly also D-benzylpenamaldic acid disulphide haptenic specificity (see Discussion).

ABSENCE OF ALLERGIC WHEAL-AND-FLARE REACTIVITY SPECIFIC FOR THE BENZYLPENICILLENIC ACID (BPE) HAPTENIC GROUP

Of the eighty-two patients tested in this study only one (C.H., Table 2) gave an unequivocal W&F response to the multivalent BPE conjugate, BPE_8–acHSA. Hapten inhibition studies could not be carried out, since C.H. reacted also to the univalent haptens, BPO–EACA and S-(phenylmercuri)-benzylpenicillenic acid (preparation given in Part I). This W&F reaction to BPE_8–acHSA may not represent specific reactivity to the BPE group as is taken up in the Discussion.

STEREOSPECIFICITY OF WHEAL-AND-FLARE RESPONSES IN THE BPO HAPTENIC SYSTEM

Solutions of the univalent haptens, the α-diastereoisomer of BPO–EACA and the equilibrium diastereoisomeric mixture of BPO–EACA were compared with regard to their abilities to specifically inhibit W&F responses in six patients with past histories of penicillin allergy who reacted to BPO_{53}–$PLL_{402}S$. In these experiments, the concentration of BPO_{53}–$PLL_{402}S$ test solutions was 10 μg./ml. (containing $1{\cdot}03 \times 10^{-5}$ moles/l. BPO) and the concentration of the BPO–EACA solutions was $1{\cdot}00 \times 10^{-3}$ moles/l. Inhibition of W&F reactions was achieved by injecting mixtures of BPO_{53}–$PLL_{402}S$ and BPO–EACA intradermally. The results in Table 3 show that in four patients, the diastereoisomeric mixture was a slightly but consistently more effective inhibitor than was the α-diastereoisomer, and in two patients the two BPO–EACA preparations were of equal effectiveness. Although small, these differences appear to be significant (see Table 3 and Materials and Methods). Stereoisomeric specificity of the W&F reaction was studied also by comparing the abilities to elicit W&F responses of BPO–$PLL_{402}S$ conjugates containing different ratios of diastereoisomeric BPO groups. The results in Table 5 show that four of seven patients gave slightly more intense W&F reactions to α-BPO_{72}–$PLL_{402}S$ (which contains mainly α-diastereoisomeric BPO groups) than to conjugates containing mixtures of the diastereoisomers of BPO (i.e. BPO_{58}–$PLL_{402}S$, prepared by mutarotation of α-BPO_{72}–$PLL_{402}S$, prepared from benzylpenicillenic acid, see Materials and Methods. The other three patients gave reactions of equal intensities to the three conjugates. Although small, these differences appear to be significant (see Table 5 and Materials and Methods).

THE EFFECT OF THE HAPTEN CARRIER ON ELICITATION OF W&F REACTIONS BY BPO-CONJUGATES

Four patients with past histories of PG allergy were skin tested simultaneously with BPO_{13}–HSA, BPO_{22}–HGG, and BPO_{53}–$PLL_{402}S$. Table 6 shows that the four patients responded differently with regard to the relative effectiveness of the BPO conjugates to elicit W&F responses. In three patients (P.C., S.L. and V.J.), BPO_{53}–$PLL_{402}S$ elicited somewhat stronger reactions than did the HGG or the HSA conjugates. Two of these three

reacted with approximately equally intense reactions to BPO_{22}–HGG and BPO_{13}–HSA whereas the other patient (S.L.) gave considerably stronger reactions to BPO_{22}–HGG. The fourth patient (I.F.) gave considerably stronger W&F reactions to the HSA conjugate than to the HGG or PLL conjugates. These observed differences in W&F reactivities

TABLE 5

COMPARATIVE EFFECTIVENESS OF DIFFERENT DIASTEREOISOMERIC MULTIVALENT BPO CONJUGATES IN ELICITING WHEAL-AND-FLARE REACTIONS

*Subject**	*Conjugate and concentration*†				
	α-BPO_{70}–$PLL_{402}S$ (α-*Diast.*)		BPO_{58}–$PLL_{402}S$ (*Diast. mixture*)	BPO_{53}–$PLL_{402}S$ (*Diast. mixture*)	
	2 μg./ml.	10 μg./ml.	10 μg./ml.	2 μg./ml.	10 μg./ml.
1 E.M.	–	10;35	9;35	–	10;35
2 E.S.	–	11;30	10;30	–	10;30
3 J.N.	9;30	10;40	8;40	8;30	9;40
4 I.P.	–	9;20	9;20	–	9;20
5 W.S.	7;10	8;20	–	6;10	8;20
6 G.B.	6;35	6;35	–	5;35	5;35
7 J.B.	10;35	11;35	10;35	7;35	10;35

* Patients with histories of penicillin allergy.

† Tests done in duplicate; mean diameters tabulated, mean deviation ±0·5–1·5 mm. (for wheal) see footnote to Table 2 for expressions of W&F responses. See footnote on page 542 for abbreviations and text for preparations and analysis of conjugates. Conjugates were non-irritant in four control subjects.

appear to be significant since tests were carried out in duplicate, three different conjugate concentrations were used, and skin areas of the arm were used which showed essentially equal skin reactivity. This heterogeneity with regard to the relative intensities of W&F reactions elicited by different BPO-conjugates can be seen also in Table 2. Here also, the

TABLE 6

EFFECT OF HAPTEN CARRIER ON ELICITATION OF WHEAL-AND-FLARE REACTIONS

*Subject**	*Conjugate and concentration*† (μg./ml.)								
	BPO_{13}–*HSA*			BPO_{22}–*HGG*			BPO_{53}–$PLL_{402}S$		
	10	100	1000	10	100	1000	10	100	1000
P.C.	1;0	6;TR	6;15	2;0	7;TR	7;15	8;15	8;15	–
S.L.	2;TR	4;TR	4;TR	5;10	7;14	7;14	8;20	9;20	–
I.F.‡	–	11;20	14;30	–	5;15	9;20	–	10;20	10;20
I.F.	12;30	12;20	13;30	–	–	–	10;20	10;20	10;20
V.J.	7;30	7;30	–	6;30	6;30	–	9;30	9;30	–

* Patients with histories of penicillin allergy.

† Skin tests done in duplicate; mean diameters tabulated, mean deviation ±1 mm. (for wheal). See footnote to Table 2 for expression of W&F reactions. –, tests not done. See footnote on page 542 for abbreviations. Reactions to TBS (diluent) and to HGG 1·0 mg/ml. were negative (2;0).

‡ Skin tests on patient I.F. were performed twice 3 days apart.

majority of patients gave more intense W&F reactions to the PLL conjugates than to BPO_{13}–HSA or BPO_{22}–HGG. About 50 per cent of the patients listed in Table 3 and all four patients listed in Table 7 were examined at 24 hours for delayed (Tuberculin) reactions to the test materials. None were observed.

EFFECT OF EXTENT OF CONJUGATION AND STRUCTURAL CHANGES IN HAPTENIC GROUPS ON W&F REACTIONS

Four patients who did not give non-specific W&F reactions to HGG were tested with multivalent BPO–HGG conjugates containing different average numbers of BPO groups per mole and with conjugates containing large numbers of allylmercaptomethylpenicilloyl (APO) and dimethoxyphenylpenicilloyl (DPO) groups per mole. Three of four patients gave less intense W&F reactions to the more lightly conjugated BPO–HGG conjugates (Table 7). Reactions to BPO_{10}–HGG were only slightly less intense than reactions to BPO_{22}–HGG, whereas reactions to BPO_3–HGG were considerably less intense than were reactions to BPO_{10}–HGG. The fourth patient (C.H.) gave equally intense reactions to all three BPO–HGG conjugates, but the intense reactions elicited in this patient might have masked differences. Table 7 shows also that three of the four patients gave somewhat weaker W&F reactions to APO_{42}–HGG and considerably weaker W&F reactions to DPO_{22}–HGG, consistent with the increasing structural differences from the BPO haptenic group. One patient (G.M.) did not cross-react to either the APO or the DPO conjugates.

TABLE 7

EFFECT OF EXTENT OF CONJUGATION ON WHEAL-AND-FLARE REACTIVITY AND CROSS REACTIONS BETWEEN ALLYLMERCAPTOMETHYLPENICILLOYL (APO), DIMETHOXYPHENYLPENICILLOYL (DPO) AND BENZYLPENICILLOYL (BPO) CONJUGATES

*Subject**	*Conjugate†*				
	BPO_3–HGG	*BPO_{10}–HGG*	*BPO_{22}–HGG*	*APO_{42}–HGG*	*DPO_{22}–HGG*
C.H.	(16;30)	(16;30)	(16;30)	(12;30)	(7;15)
G.M.	4;0	7;10	10;30	3;0	3;0
G.Mc.	7;35	12;35	13;35	8;30	5;18
M.Q.	6;25	9;28	10;25	7;20	3;0

* Patients with histories of penicillin allergy.

† See footnote to Table 2 for expression of W&F reactions. See footnote on page 542 for abbreviations. Concentrations at 2·0 mg./ml. except for results in parentheses which are of tests with conjugates at 0·2 mg./ml. Reactions to TBS and HGG solution 2·0 mg./ml. were negative (3;0).

CLINICAL DATA

Some pertinent clinical data with regard to the forty-one patients with acceptable histories of allergic reactions to PG are given in Tables 1, 2 and 8. Overall, 29 per cent (twelve of forty-one) gave unequivocal W&F reactions to multivalent BPO-conjugates. Only one patient (M.B., Table 4) reacted to other skin test reagents but not to BPO-conjugates. The percentage of positive W&F reactions to BPO-conjugates was higher in patients who had recent allergic reactions to PG than in patients who had allergic reactions in the distant past.

The percentage of patients with acceptable histories of PG allergy exhibiting W&F reactivity was always below 50 per cent. Also three patients who developed typical late diffuse urticarial reactions to PG, and the majority of patients who have had recent allergic reactions to PG do not demonstrate W&F reactivity to any of the presently known elicitors of W&F reactions in PG-hypersensitivity.

DISCUSSION

The foregoing results demonstrate that the benzylpenicilloyl (BPO) group,*† is the major haptenic determinant of wheal-and-flare (W&F) allergic skin reactivity in human beings hypersensitive to benzylpenicillin (PG). These results are consistent with the results of previous studies in human beings (Levine and Ovary, 1961; Parker *et al.*, 1962) and in experimental animals (Levine, 1960a, 1964; Levine and Ovary, 1961; De Weck, 1962).

TABLE 8

CLINICAL DATA ON PATIENTS WITH ACCEPTABLE HISTORIES OF PENICILLIN ALLERGY

	*No. of positive reactors**	*No. of negative reactors*
Time elapsed between allergic reaction and skin testing		
Less than 1 month	5	9
1–3 months	1	2
3–12 months	3	3
More than 12 months	3	15
Clinical form of allergic reaction		
Immediate urticarial†	2	4
Late urticarial†	6	14
Urticarial with arthritis	2	2
Maculo-papular	1	7
Other	1‡	2§

* Wheal-and-flare reactions to multivalent BPO conjugates, or to other test reagents listed in Table 2.

† Immediate urticarial reactions occurred in four patients within 30 minutes and in two patients 1½ and 2 hours after penicillin administration. Late urticarial reactions occurred between 1 and 7 days after penicillin in seventeen cases and at 2 weeks after PG administration in three cases. Four patients in these groups also had angioedema.

‡ Erythema multiforme and nodosum.

§ One immediate hypotension and vomiting; one immediate angioedema.

Only one of the twelve patients exhibiting W&F reactivity to BPO-conjugates (and none of the other patients) gave a weak W&F reaction to the multivalent benzylpenicillenic (BPE) conjugate, BPE_8–acHSA. Since this conjugate contained at least trace amounts of BPO groups as a contaminant (see Levine, 1964), and the patient (C.H., Table 2) gave strong W&F reactions even to lightly coupled BPO-conjugates (i.e. BPO_3–HGG, Table 7), it is not clear whether W&F reactivity was specific for the BPE group or for the BPO

* The BPO haptenic group is covalently bound to proteins predominantly through amide linkages with lysine ε-NH_2 groups (Levine, 1961, 1963; Levine and Ovary, 1961). Rabbit anti-BPO antibodies show immunological specificity also for the lysine residue (Levine, 1962, 1963). BPE and PSH haptenic groups are covalently bound to proteins through disulfide links with cysteine residues (Levine, 1961; Levine and Ovary, 1961).

† With regard to the stereospecificity of W&F reactions elicited by multivalent BPO-conjugates, the data in Tables 3 and 5 may be interpreted as indicating that specificity is directed mainly to the α-diastereoisomer but also to one or more of the other three possible stereoisomers. This interpretation is based on the observations that the equilibrium diastereoisomeric BPO–EACA preparation was a slightly more effective hapten inhibitor than was the α-diastereoisomeric BPO–EACA, whereas the α-diastereoisomeric multivalent BPO conjugate was slightly more effective than were multivalent conjugates containing diastereoisomeric mixtures of BPO groups. Identical results were observed in guinea-pigs passively sensitized with rabbit anti-PG sera, and these results are discussed in detail in Part I (Levine, 1964).

contaminant contained by BPE_8–acHSA. Hapten inhibition experiments could not be done as the univalent haptens elicited specific W&F reactions in this patient (probably because of their reactions with tissue proteins to form multivalent D-penicillamine disulphide conjugates or D-benzylpenamaldic acid disulphide conjugates, see below). These results are in contrast to those of Parker *et al.* (1962) who reported that twenty-four of forty-six (52 per cent) patients giving W&F responses to a multivalent BPO–polylysine conjugate gave positive reactions also to a multivalent BPE-conjugate which these authors reported to be free from contaminating BPO groups. Although Parker's results and the results presented here cannot be reconciled at the present time, it is possible that the multivalent BPE-conjugate used by Parker *et al.* contained haptenic impurities which are structurally sufficiently similar to the BPO group to cross react immunologically, but which do not undergo the penamaldate reaction used for assay of BPO groups, e.g. benzylpenicilloyl sulphone groups. The BPE-conjugate (BPE_8–acHSA) used in this study appears to be capable of eliciting W&F reactions specific for the BPE group, as it was demonstrated to be capable of eliciting strong cutaneous anaphylactic reactions specific for the BPE group in guinea-pigs passively sensitized with rabbit anti-BPE sera (Levine, 1964). Accordingly the present findings indicate that BPE-specific W&F hypersensitivity is at most a comparatively rare event. Also, anti-BPE antibodies were not found in rabbit anti-PG sera (Levine and Ovary, 1961; Levine, 1964).

Two patients gave specific W&F reactions to a multivalent D-penicillamine disulphide (PSH) conjugate PSH_{10}–HSA, and accordingly appear to show PSH haptenic specificity. Consistent with this view is the finding that these patients (M.B. and C.H., Tables 2 and 4) reacted also to PG* and to sodium benzylpenicilloate. These compounds can probably react with cystine–disulphide linkages of tissue proteins to form finally† in the skin test site, multivalent PSH conjugates (Levine, 1960a, b). PSH-specificity of W&F reactions elicited by PG and by benzylpenicilloate was confirmed in a third patient by specific hapten inhibition of these W&F reactions using D-penicillamine-cysteine mixed disulphide as the univalent hapten. The inability of D-penicillamine to elicit W&F reactions in these patients may be due to the specific hapten inhibition activity of unreacted or oxidized D-penicillamine remaining in the skin test site. PSH-specificity has been demonstrated also for allergic contact dermatitis to PG in guinea-pigs and in human beings (Levine, 1960a).

Two considerations may be of clinical importance to patients with PSH-specific penicillin hypersensitivity: (1) Aside from the known presence of PG in some milk products (Welch, Jester and Burton, 1956), the probable presence also of benzylpenicilloate, a hydrolysis product of PG (Levine, 1960c), may induce PSH-specific hypersensitivity or provoke PSH-specific clinical allergic reactions. (2) Penicillinase hydrolyses PG to penicilloate (Becker, 1956), and accordingly would not benefit patients with PSH-specific allergic reactions to PG. Penicillinase may even intensify these allergic reactions by increasing the concentration of benzylpenicilloate (and subsequently PSH-conjugates) in the tissues.

* Not all W&F reactions to PG are due to PSH specificity. In another study, Siegel and Levine (1963) reported that human skin sites passively sensitized with sera from three patients with recent immediate systemic allergic reactions to PG gave W&F reactions to PG, but not to benzylpenicilloate nor to multivalent BPO, BPE or PSH conjugates. The antigenic specificities of these reactions have not been determined. The rare occurrence of W&F reactivity to PG in patients with late allergic reaction to PG seen in this study, has been observed also by others (Tuft, Gregory and Gregory, 1955; Smith, 1957). It appears to be due mainly to the slow rate of chemical reaction of PG with proteins in the skin test site to form multivalent BPO-conjugates. This is discussed in detail in the previous paper (Levine, 1964).

† The reaction proceeds through the intermediate D-penamaldic acid-cysteine mixed disulphide (Levine, 1960a, b) which may also be a haptenic antigenic determinant in patients hypersensitive to the PSH group.

The three patients manifesting PSH-specific hypersensitivity had unusual clinical allergic reactions: C.H., immediate systemic reaction; M.B., prolonged recurrent urticaria; E.N., prolonged recurrent urticaria and anaphylaxis. Although the unique ability of these three patients to develop skin-sensitizing antibodies specific for the PSH group may be due to environmental factors such as prolonged contact with PG (e.g. in patients who consume large amounts of milk products), it is possible that this unusual immune response to PG may reflect a unique constitutional ability to respond with the formation of skin-sensitizing antibodies to trace amounts of antigen. Further studies on this group of patients would be of considerable theoretical interest.

Rabbit anti-BPO antibody combining sites have been shown previously to be sufficiently large to encompass the entire large BPO haptenic group and adjoining structural areas of the carrier protein of the immunizing conjugate (i.e. carrier specificity (Levine, 1963)). Similar results were obtained in the present study for anti-BPO-antibodies mediating W&F reactions in humans. The data in Table 7 which show that highly coupled HGG conjugates of the allylmercaptomethylpenicilloyl and the dimethoxyphenylpenicilloyl haptenic groups elicited weaker W&F reactions than did BPO_{22}–HGG indicate that considerable specificity is directed toward the penicilloyl side chain. These observations indicate that a considerable portion of skin sensitizing anti-BPO antibodies are specific for the entire bifunctional BPO haptenic group. The observed heterogeneity in the extent of cross-reactions among the four patients (Table 7) may be provisionally interpreted as due to differences among the W&F mediating antibodies produced by the four patients with regard to average antibody–hapten binding affinities.

The data in Table 6 show that one patient, I.E., gave considerably stronger W&F reactions to BPO_{13}–HSA than to BPO_{22}–HGG and to BPO_{53}–$PLL_{402}S$, whereas the three other patients gave the most intense W&F reactions to BPO_{53}–$PLL_{402}S$. These data indicate that BPO-specific W&F allergic reactivity, in patient I.F., was specific also for structural areas of the HSA carrier (carrier specificity), since the greater effectiveness of BPO_{13}–HSA was not due to non-specific differences among the three BPO-conjugates (e.g. in numbers of BPO groups per mole, in extents of steric inhibition to antibody–hapten binding by carrier protein structures, or in rates of diffusion in skin test sites).

The greater W&F reactivity of BPO conjugates of PLL (compared with conjugates of HGG or HSA) in the majority of patients tested (Tables 2 and 6) may be due to the open, random-coil structure of BPO–PLL(S) conjugates, which would permit a minimum of steric-interference to hapten–antibody binding by carrier structures adjoining the point of attachment of hapten (see Discussion, Part I (Levine, 1964). These observations may be interpreted as indicating that the antibodies mediating these W&F reactions in the majority of patients exhibit carrier specificity for an unidentified autologous carrier protein which is neither HSA nor HGG. The alternative possibility that these antibodies do not exhibit carrier specificity at all is unlikely, since carrier specificity has been demonstrated for anti-hapten antibodies in many situations where it has been sought adequately (Haurowitz, 1942; Eisen, Carsten and Belman, 1954; Buchanan-Davidson *et al.*, 1959; Levine, 1963) and may prove to be a general rule. Consistent with this interpretation, the absence of delayed reactions to BPO-conjugates observed in this study may be due, in part, to the possibility that the BPO-protein conjugate(s) which induced hypersensitivity was not used for testing. The marked dependence of the delayed reaction on carrier specificity is known (Benacerraf and Gell, 1959; Benacerraf and Levine, 1962).

This inference that human anti-hapten antibodies which mediate allergic reactions to drugs manifest specificity also for autologous carrier proteins (carrier specificity) would mean that drug allergies are in part auto-immune diseases. This view may explain some peculiarly localized allergic reactions to drugs, although the precise clinical significance of carrier specificity is not yet known.

Two clinical considerations merit discussion. Firstly, from the foregoing data and from the findings of Siegel and Levine (1963), it appears that patients should be skin-tested with both a multivalent BPO-conjugate and with PG in order to detect W&F reactivity in the maximum number of patients. Succinylated BPO–polylysine conjugates appear at present to be the skin test reagents of choice, as they are at least as effective elicitors as are BPO–protein conjugates. Also, BPO–PLL(S) conjugates are not antigenic in guinea-pigs (B. B. Levine, unpublished) and accordingly may not be antigenic in man.

Secondly, only 15–40 per cent of patients with acceptable past histories of allergic reactions to PG demonstrated W&F skin reactivity to any of the known effective skin test reagents pertinent to PG hypersensitivity. Many of the non-reactors of this group experienced allergic reactions within 1 month prior to skin testing, and it is unlikely that a considerable proportion of the non-reactors had not, in fact, experienced an allergic reaction to PG. Indeed, several patients who experienced typical late urticarial reactions after treatment with PG alone, consistently gave negative W&F reactions to the test reagents during and after the allergic reactions. Accordingly, while the skin sensitizing antibodies which mediate the W&F reaction may play a role in the pathogenesis of some cases of PG allergy, other immunological mechanisms which do not involve the participation of skin-sensitizing antibodies would appear to be operative in other cases. Also, a considerable percentage of patients who have received PG in the past and who demonstrate BPO-specific W&F hypersensitivity, may tolerate injections of PG (at least in comparatively small dosage) without experiencing a clinical allergic reaction, although some of these patients may experience an immediate systemic allergic reaction on administration of PG (Parker *et al.*, 1962). Accordingly, the clinical significance of W&F reactivity in the PG system cannot be clearly defined in the absence of more complete knowledge concerning the immunological and non-immunological mechanisms involved in the occurrence of allergic tissue damage in penicillin allergy.

ACKNOWLEDGMENTS

This work was supported by the Health Research Council of the City of New York under Contract No. U-1297.

B.B.L. was a Recipient of Investigatorship of the Health Research Council of the City of New York under Contract No. I-240.

REFERENCES

Becker, R. M. (1956). 'Effect of penicillinase on circulating penicillin.' *New Engl. J. Med.*, **254**, 952.

Benacerraf, B. and Gell, P. G. H. (1959). 'Studies on hypersensitivity. I. Delayed and Arthus type skin reactivity to protein conjugates in guinea pigs.' *Immunology*, **2**, 53.

Benacerraf, B. and Levine, B. B. (1962). 'Immunological specificity of delayed and immediate hypersensitivity reactions.' *J. exp. Med.*, **115**, 1023.

Buchanan-Davidson, D. J., Dellert, E. E., Kornguth, S. E. and Stahman, M. A. (1959). 'Immunochemistry of synthetic polypeptides and polypeptidyl proteins. II. Quantitative studies on the modified proteins.' *J. Immunol.*, **83**, 543.

De Weck, A. L. (1962). 'Studies on penicillin hypersensitivity. I. The specificity of rabbit anti-penicillin antibodies.' *Int. Arch. Allergy*, **21**, 20.

Eisen, H. N., Carsten, M. E. and Belman, S. (1954). 'Studies on hypersensitivity to low-molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction.' *J. Immunol.*, **73**, 296.

GROVE, D. G. and RANDALL, W. H. (1955). *Assay Methods of Antibiotics*, p. 14. Medical Encyclopedia Inc., New York.

HAUROWITZ, F. (1942). 'Separation and determination of multiple antibodies.' *J. Immunol.*, **43**, 331.

LEVINE, B. B. (1960a). 'Studies on the mechanism of the formation of the penicillin antigen. I. Delayed allergic cross-reactions among penicillin G and its degradation products.' *J. exp. Med.*, **112**, 1131.

LEVINE, B. B. (1960b). 'Formation of penicillamine-cysteine mixed disulfide by reaction of D-benzyl-penicilloic acid with cysteine.' *Nature (Lond.)*, **187**, 940.

LEVINE, B. B. (1960c). 'Degradation of benzyl-penicillin at pH 7·5 to D-benzylpenicilloic acid.' *Nature (Lond.)*, **187**, 939.

LEVINE, B. B. (1961). 'Studies on the formation of the penicillin antigen. II. Some reactions of D-benzyl-penicillenic acid in aqueous solution at pH 7·5.' *Arch. Biochem. Biophys.*, **93**, 50.

LEVINE, B. B. (1962). 'N-(α-D-penicilloyl) amines as univalent hapten inhibitors of antibody dependant allergic reactions to penicillin.' *J. med. pharm. Chem.*, **5**, 1025.

LEVINE, B. B. (1963). 'Studies on the dimensions of the rabbit anti-benzylpenicilloyl antibody combining sites.' *J. exp. Med.*, **117**, 161.

LEVINE, B. B. (1964). 'Studies on the immunological mechanisms of penicillin allergy. I. Antigenic specificities of guinea-pig skin sensitizing rabbit anti-benzylpenicillin antibodies.' *Immunology*, **7**, 527.

LEVINE, B. B. and OVARY, Z. (1961). 'Studies on the mechanism of formation of the penicillin antigen. III. The N-(D- α-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G.' *J. exp. Med.*, **114**, 875.

PARKER, C. W., SHAPIRO, J., KERN, M. and EISEN, H. N. (1962). 'Hypersensitivity to penicillenic acid derivatives in human beings with penicillin allergy.' *J. exp. Med.*, **115**, 821.

SIEGEL, B. B. and LEVINE, B. B. (1963). 'Antigenic specificities involved in the immediate systemic reactions to penicillin (Abstr.). Program of Amer. Acad. Allergy, 19th Annual Meeting, March 1963, p. 31. (1964) *J. Allergy* (In press).

SMITH, V. M. (1957). 'Fatal reactions to penicillin, evaluation of a test for sensitivity.' *New Engl. J. Med.*, **257**, 447.

TABACHNICK, M., EISEN, H. N. snd LEVINE, B. B. (1954). 'A new mixed disulfied: penicillamine-cysteine.' *Nature (Lond.)*, **174**, 701.

TUFT, L., GREGORY, D. C. and GREGORY, J. (1955). 'Evaluation of skin test methods employed in the diagnosis of penicillin allergy.' *Amer. J. Med.*, **230**, 370.

WELCH, H., JESTER, W. R. and BURTON, J. M. (1956). 'Antibiotics in fluid market milk. Third nationwide survey.' *Antibiot. et Chemother.*, **6**, 369.