

STUDIES ON THE MECHANISM OF THE FORMATION OF THE
PENICILLIN ANTIGEN

I. DELAYED ALLERGIC CROSS-REACTIONS AMONG PENICILLIN G AND
ITS DEGRADATION PRODUCTS

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PLATES 94 AND 95

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INTRODUCTION

The problem of penicillin allergy is of considerable importance in both clinical medicine and theoretical immunology. Up to now investigation in penicillin allergy has been hampered by a lack of knowledge of the chemical reactions which occur between penicillin and tissue proteins to form the "penicillin antigen," as well as by lack of knowledge as to the identity of the specific allergic determinant groups of this antigen.

It is generally believed that low molecular weight allergens induce the allergic state and elicit allergic reactions by first combining through a covalent bond with tissue proteins to form the complete antigen. This view is based on the classic studies of Landsteiner, Jacobs, Chase, Gell, and Eisen (1-6), who showed that only those compounds which could react irreversibly with proteins or with protein model compounds could sensitize or elicit an allergic reaction.

The great majority of clinical sensitizers, however, such as penicillin, the sulfonamides, and aspirin have not been shown to react irreversibly with proteins. In these cases it is plausible to consider that either a trace contaminant, or a degradation or metabolic product may be the actual proantigen¹ responsible for allergy to the parent compound. With regard to this consideration, Landsteiner and Di Somma reported that guinea pigs with allergic contact dermatitis to picric acid gave allergic cross-reactions to its reduction product, picramic acid (7). Also, Mayer has shown that human beings and guinea pigs with allergic contact

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¹ By "proantigen" is meant a low molecular weight chemical allergen that reacts irreversibly with tissue protein to form the complete antigen. This term was introduced by Gell *et al.* (3).

dermatitis to *p*-phenylenediamine cross-reacted to its oxidation products *p*-benzoquinone and *p*-quinonediimine (8).

Penicillin G (PG)² has not been demonstrated to undergo irreversible reaction with proteins or with low molecular weight amines, thiols, or disulfides in neutral aqueous solution (9, 10). In view of the reversible binding of salts of PG to serum albumin (11), it might be considered that PG acts as an allergen by virtue of its reversible binding to tissue proteins. There is no clear evidence in favor of this possibility, and some data have been obtained which indicate that such easily dissociable complexes are incapable of inducing the allergic state (6).

In the present work, the patterns of allergic cross-reactivity among PG and its degradation products were studied using allergic contact dermatitis in the guinea pig as the test system. Three degradation products of PG, D-benzylpenicillenic acid (BPE) D-penicillamine (PSH), and D-benzylpenicilloic acid (BPO) were found to cross-react with PG. By correlating these immunologic data with data on the *in vitro* degradation pathways of PG and on the chemical reactivities of its degradation products in aqueous solution at pH 7.5 (12-15), a schema has emerged which describes the chemical reactions which may be involved in the *in vivo* formation of the penicillin antigen. This schema is presented in the Discussion.

Degradation Pathways of PG.—The only data available on the *in vivo* degradation of PG are those of Walshe (16) who isolated a compound with some of the properties of PSH³ from the urine of patients given large doses of PG. *In vitro*, PG is degraded as follows (Text-fig. 1):—

In pH 7.5 phosphate buffer solution *in vitro*, PG (Ia) is degraded to yield mainly a mixture of diastereomers of BPO (II) (12). This reaction exhibits first order kinetics and proceeds at a rate of 1.2 per cent per hour at 37° (17). The appearance of a small quantity of BPE (III) was detected in this degrading PG solution (12).

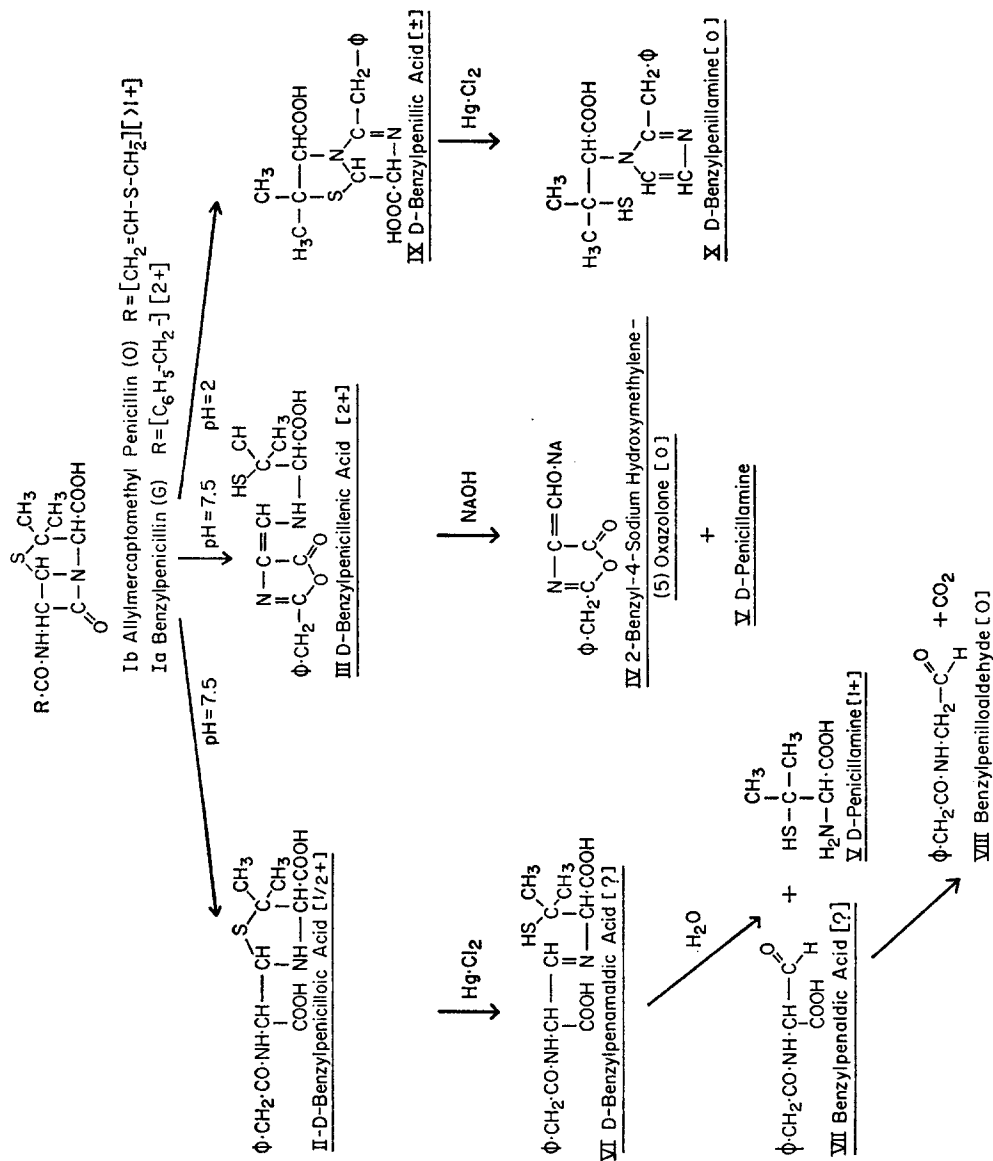
In aqueous solution, BPO is further degraded by mercuric chloride to PSH (V), benzylpenilloaldehyde (VIII), and CO₂ (18 a). This degradation proceeds presumably through the intermediate D-benzylpenamaldic acid (VI), which is a Schiff base and readily decomposes to PSH and benzylpenaldic acid (VII) (18 b, 19 a). Free benzylpenaldic acid is unstable, and is immediately decarboxylated to benzylpenilloaldehyde (18 c). BPE is degraded to 2-benzyl-4-sodium hydroxymethylene-(5)-oxazolone (BSO) (IV) and PSH by aqueous sodium hydroxide (19 b).

At alkaline pH, PG is hydrolyzed to the α -diastereomer of BPO (18 d, 19 c) and at

² The following abbreviations are used:

PG = (penicillin G); BPE = (D-benzylpenicillenic acid);
 BPO = (D-benzylpenicilloic acid); PSH = (D-penicillamine);
 BSO = (2-benzyl-4-sodium hydroxymethylene-(5)-oxazolone);
 PSSCy = (D-penicillamine-cysteine mixed disulfide).

³ This compound may be penicillamine disulfide. The identifying tests performed on this compound, paper chromatography, bromine oxidation, and elemental analysis would not distinguish PSH from penicillamine disulfide.



TEXT-FIG. 1. The *in vitro* degradation pathways of penicillin G (PG), and summary of the allergic reactivities of the degradation products of PG. In parentheses, following the name of the compound, is placed the average intensity of the delayed contact allergic reaction given by a 0.1 M solution of the compound in a PG-sensitized guinea pig which reacts 2+ to a 0.1 M solution of PG. See text for methods of sensitization and elicitation.

acid pH, PG rearranges to D-benzylpenillic acid (IX) (19 *d*). D-Benzylpenillic acid is further degraded to D-benzylpenillamine (X) by aqueous mercuric chloride (19 *e*). Detailed discussions of the chemistry of penicillin and its degradation products are contained in references 12 to 20.

EXPERIMENTAL AND RESULTS

Materials.—Crystalline sodium penicillin G (NaPG) (1650 units per mg.) was generously furnished by Pfizer Laboratories, Brooklyn, New York. Crystalline potassium penicillin G (KPG) (lot E-7941) was obtained from Bristol Laboratories, Syracuse, New York. It bio-assayed at 1600 units per mg. and was 99 per cent pure; the remainder was penicillins K and F (manufacturer's assay). Crystalline sodium penicillin O (cer-o-cillin, sodium, lot KM-008) was furnished by the Upjohn Co., Kalamazoo, Michigan. It contained 0.06 to 0.10 per cent PG as an impurity (manufacturer's assay). D-Penicillamine·HCl·½ H₂O, (α)_D²⁰ = -49.7° (1 per cent in 1 N NaOH), and L-penicillamine·HCl·½ H₂O, (α)_D²⁰ = +60.2° (1 per cent anhydrous free amine in 1 N NaOH), were purchased from California Corporation for Biochemical Research, Los Angeles, California. These crystalline materials were prepared by synthesis and were over 99 per cent pure (manufacturer's assay). Both compounds gave strongly positive nitroprusside reactions. D-Cysteine·HCl, (α)^P = -3.4° (2 per cent in water), was purchased from California Corporation. It exhibited a strongly positive nitroprusside reaction.

N-Acetyl-D-penicillamine was prepared from PSH by reaction with acetic anhydride (19 *f*), and was crystallized twice from water. M.p. = 192–193° (micro., dec.).⁴ Its nitroprusside reaction was strongly positive, and it ran as one spot (R_f = 0.79) on phenol-water descending paper chromatography. D-Penicillamine disulfide was prepared by air oxidation of PSH (19 *g*), and was crystallized from water-ethanol and finally from water-acetone. M.p. = 204–205° (cap., dec.). Its nitroprusside reaction was negative, and it ran as one spot (R_f = 0.65) on phenol-water descending paper chromatography. Monosodium D- α -benzylpenicilloate was prepared by alkaline hydrolysis of NaPG (19 *c*) and was crystallized from water and finally from water-acetone. M.p. = 154–155° (micro., dec.); (α)_D²⁰ = +125° (0.772 per cent in 0.2 M pH 7.5 phosphate buffer). D-Benzylpenillic acid was prepared by rearrangement of NaPG in acid aqueous solution (19 *d*) and recrystallized twice from water. M.p. = 185–186° (cap., dec.); λ_{\max} 275 m μ (shoulder), ϵ = 6500.

D-Benzylpenillamine·HCl was prepared by mercuric chloride degradation of D-benzylpenillic acid followed by H₂S decomposition of the resulting mercuric mercaptide of D-benzylpenillamine (19 *e*). It was crystallized twice from acetone-ether. M.p. = 172–173° (cap., dec.). Its nitroprusside reaction was strongly positive. Benzylpenilloaldehyde was prepared by synthesis (19 *h*), and crystallized from acetone and finally from benzene. M.p. = 112–113° (cap.). Its 2,4-dinitrophenylhydrazone derivative prepared by the method of Shriner and Fuson (21) showed the expected melting point of 194–195°C. (micro., dec.) (19 *i*).

BPE was prepared by rearrangement of PG with mercuric chloride followed by H₂S decomposition of the resulting mercuric mercaptide of BPE. Its preparation is described in detail in another report (13). Its nitroprusside reaction was strongly positive. It exhibited the expected ultraviolet absorption maximum at λ 322 m μ with ϵ = 25,600, which corresponds to 96 per cent purity⁵ (see below under BPE content of test compounds). BSO² was prepared

⁴ Melting points were determined with a capillary melting point apparatus (cap.), or with a Fisher-Johns microblock apparatus (micro.) and are uncorrected.

⁵ Approximately 11 per cent of the BPE content of the preparation is present in the oxidized (disulfide) form. BPE disulfide appears to be unreactive in neutral aqueous solution (13).

by alkaline degradation of BPE (19 *b*), and was crystallized from water and finally from an ethyl acetate-methanol mixture. M.p. = 236–237° (micro., dec.). In 0.01 M NaOH solution, it exhibited the expected ultraviolet absorption peaks at λ_{\max} 299 m μ , ϵ = 18,300, and λ_{\max} 240 m μ , ϵ = 5,400 (19 *j*).

Tween 80, tris (polyoxyethylene) sorbitan monooleate was kindly donated by the Atlas Powder Corporation, New York. Other chemicals were of reagent grade.

TABLE I
*Purity of Test Compounds**

Test compound	Penicillin G content	D-Penicillamine content	D-Benzylpenicillenic acid content
	Per cent by weight	Molar per cent [‡]	Molar per cent [‡]
1. Potassium penicillin G	99 [§]	0	0
2. Sodium penicillin O	0.06–0.10	0	0.1 per cent [¶]
3. D-Penicillamine·HCL· $\frac{1}{2}$ H ₂ O	0	99 ⁺	0
4. Monosodium D- α -benzylpenicilloate	0	0	0
5. D-Benzylpenillic acid	0	0	0
6. D-Benzylpenillamine·HCL	0	0	0
7. D-Benzylpenicillenic acid	0.02	0.1 maximum	96 ^{**}
8. Benzylpenilloaldehyde	0	0	0
9. 2-Benzyl-4-sodium hydroxymethylene-(5)-oxazolone	0	0	0

* See text for analytical methods and limits of sensitivity.

[‡] Mols D-penicillamine or D-benzylpenicillenic acid per 100 mols test compound.

[§] The other 1 per cent is composed of penicillins K and G (manufacturer's assay).

^{||} Manufacturer's assay.

[¶] Probably present as allylmercaptomethylpenicillenic acid.

^{**} Approximately 11 per cent of the BPE present in the preparation is in the disulfide form (13).

Purity of Test Compounds.—With the exception of BPE, the test compounds were all crystalline and exhibited sharp melting points. BPE, PSH, and PG impurities in the test compounds were assayed quantitatively as these three compounds were found to exhibit strong allergic activity in the PG contact dermatitis system. The analytical procedures are described below, and the results are shown in Table I. The test compounds contained no detectable PG, BPE, or PSH impurity except for sodium penicillin O which contained 0.06 to 0.10 per cent PG and 0.1 per cent "penicillenic acid,"⁶ and BPE which contained 0.02 per cent PG and a maximum PSH content of 0.1 per cent (see below under PSH content of test compounds).

Penicillin Content of Test Compounds.—The quantity of penicillin impurity in the test compounds was determined by bioassay of 1 mg. per ml. solutions of these materials in 0.01

⁶ This is probably D-allylmercaptomethylpenicillenic acid.

m pH 6 phosphate buffer.⁷ A cylinder plate method with *Sarcina lutea* as the test organism was employed (22). This method can detect less than 0.01 unit per ml. of penicillin activity, or less than 0.01 per cent by weight of PG impurity. The test substances did not interfere with the analysis, as added PG was detectable.

BPE Content of Test Compounds.—The amount of BPE impurity of the test compounds was assayed by measuring the optical density (O.D.) at λ_{\max} 322 $m\mu$ of freshly prepared 1.00×10^{-2} M solutions of the test compounds in 0.1 M pH 7 phosphate buffer. Crystalline, synthetic D, L-benzylpenicillenic acid exhibits an ultraviolet absorption peak in 95 per cent ethanol at λ 322 $m\mu$ with $\epsilon = 26,600$ (23). This value is taken as the molar extinction coefficient of BPE. BPE exhibits the same molar extinction coefficient in pH 7 buffer solution as it does in 95 per cent ethanol solution (13). Accordingly, a test compound containing more than 0.01 molar per cent of BPE impurity (mols BPE per 100 mols test compound), would exhibit an O.D. at λ_{\max} 322 $m\mu$ of more than 0.027. In a typical analysis, a freshly prepared 1.00×10^{-2} M solution of KPG exhibited an O.D. at λ 322 $m\mu$ of 0.025 and no absorption peak was observed. KPG therefore contains less than 0.01 molar per cent of BPE impurity. O.D. measurements were made with the Beckman model DU spectrophotometer using 1 cm. matched quartz cuvettes.

PSH Content of Test Compounds.—This was determined by descending *N*-butanol-water-acetic acid paper chromatography on Whatman number 3 paper of 0.0256 millimol quantities of the test compounds. In a typical analysis, 9.5 mg. of KPG (0.0256 millimol) was chromatographed simultaneously with a mixture of 9.5 mg. KPG and 5.0 micrograms of PSH·HCl· $\frac{1}{2}$ H₂O (0.0256 micromol). In order to gain maximum separation of the compounds, chromatography was allowed to continue until the solvent front was almost at the end of the paper, about 45 cm. After color development with ninhydrin, the KPG-PSH mixture showed a distinct spot at $R_f = 0.13$ (PSH), well separated from the penicillin streak starting at $R_f = 0.21$. KPG alone showed no PSH spot. Thus, there is less than 0.1 molar per cent PSH impurity in KPG. In the case of monosodium D- α -benzylpenicilloate, the PSH spot was not so well separated from the penicilloate streak, but the absence of PSH could still be determined. On paper chromatography, BPE exhibited a spot with the same R_f as PSH which overlapped the BPE streak and corresponded in color intensity to 0.1 per cent PSH. It is possible that this spot is an artifact, since BPE is prepared by a method which should eliminate virtually all PSH from it (13).

In order to determine whether the 0.02 per cent PG impurity and the 0.1 per cent PSH impurity present in the BPE preparation are responsible for the strong allergic cross-reactions which occur among BPE, PSH, and PG (Table V), PSH-sensitized guinea pigs were tested with 0.1 M and 1.0×10^{-4} M solutions of PSH, and PG-sensitized animals were tested with 0.1 M, 1.0×10^{-4} M, and 2.0×10^{-5} M solutions of KPG. PSH-sensitized guinea pigs which exhibited 2+ and 3+ reactions to 0.1 M PSH gave only occasional \pm reactions to 10^{-4} M PSH. PG-sensitized animals which reacted 2+ and 3+ to 0.1 M KPG exhibited negative reactions to 2×10^{-5} M KPG and gave occasional \pm reactions to 10^{-4} M KPG. Thus, the trace PSH and PG impurities present in the BPE preparation cannot account for the strong cross-reactions observed to occur among BPE, PG, and PSH (Table V). Similarly, the trace PG impurity in the sodium

⁷ I wish to thank Dr. H. Baker and Mr. O. Frank of the Department of Chemistry, the Mount Sinai Hospital, New York, for their help in performing the bioassays.

penicillin O preparation cannot account for the strong allergic cross-reactions which occur between penicillins O and G (Table IX). Since BPE-sensitized guinea pigs which reacted 2+ and 3+ to 0.1 M BPE exhibited only occasional \pm reactions to 10^{-4} M BPE, the 0.1 per cent "penicillenic acid" impurity present in sodium penicillin O cannot account for the cross-reactions between penicillins O and G (Table IX).

*Sensitization of Guinea Pigs to PG and Its Degradation Products.*⁸—Male albino guinea pigs weighing 300 to 600 gm. were used for these experiments. They were maintained on Rockland guinea pig pellets and water *ad libitum*, and given fresh lettuce once a day. Sixty guinea pigs were sensitized with PG by six percutaneous applications of a 0.1 M solution of KPG in a solvent composed of 95 per cent ethanol, methylcellosolve,⁹ and tween 80 (45:45:10 by volume) (ECT). A freshly prepared solution was used for each application. Each application consisted of 0.05 ml. spread with a fire-polished glass rod into a 2 cm. circular area on the dorsal skin surface clipped free of hair. The same skin area was used for each application since the impression was gained that a greater incidence of sensitization was obtained by this method than by using different skin sites for each application. The patterns of cross-reaction were the same for animals sensitized by each of these two methods. The applications were made on alternate days 3 times a week for 2 weeks. After a 14 day rest period, the animals were patch-tested for hypersensitivity with 0.1 M KPG in ECT solvent. Sensitization of groups of ten to forty guinea pigs with PSH, BPE, benzylpenilloaldehyde, BSO, and BPO was accomplished by the same procedure using 0.1 M solutions of the sensitizer. These sensitizers were all dissolved in ECT solvent except for BPO which was dissolved in a mixture of H₂O, methylcellosolve, and tween 80 (45:45:10 by volume) (WCT). After a 14 day rest period, the animals were tested for hypersensitivity with a 0.1 M solution of the sensitizer.

In Table II is indicated the percentage of guinea pigs which became hypersensitive to PG and its degradation products. Guinea pigs reacting 1+ and greater to 0.1 M solution of the sensitizer in ECT or WCT were considered sensitized. PSH, BPE, and benzylpenilloaldehyde sensitized 85 to 90 per cent of the animals, whereas PG, BPO, and BSO sensitized 50 per cent.

Elicitation of Allergic Reactions.—The hair of the dorsal skin surface was closely clipped with an electric small animal clipper and a number 0000 blade.¹⁰ Care was taken to minimize skin trauma during clipping. Twenty-four hours after clipping, 0.04 ml. of the test solution was gently spread into a circular area of 1.5 cm. diameter with a thin, fire-polished glass rod. In many cases, multiple simultaneous skin testing was done, up to eight tests at a single time. This could be done since the skin area employed, a rectangular area of the dorsal skin surface from below the nuchal fat pad to the middle of the sacrum and 5 cm. from the midline on either side, showed no consistent differences in skin reactivity. However, it was found that multiple simultaneous skin testing slightly diminishes the intensity of the individual reaction.

⁸ Some of the early experiments on the sensitization of guinea pigs to PG and PSH were done at the Institute of Industrial Medicine, New York University Postgraduate Medical School, in collaboration with Dr. Herman N. Eisen presently of the Washington University School of Medicine, St. Louis, Missouri. I wish to thank Dr. Eisen for suggesting the use of tween 80.

⁹ Ethylene glycol monomethyl ether.

¹⁰ Purchased from Oster Products, Inc., New York.

The tests were read 48 hours later, 4 hours after depilation with nair, a commercial depilatory. Reactions at 48 hours were recorded exclusively since it was found that both strong and weak allergic reactions were at their maximum intensities at that time. The readings were graded as follows:—

- 0, no visible reaction or faint erythematous stippling.
- ±, small areas of erythema in the test circle.
- 1+, a homogeneous, sharply demarcated, slightly raised, mildly erythematous area.
- 2+, a homogeneous, raised, moderately erythematous area.
- 3+, a homogeneous, raised, intensely erythematous area.

The solvent mixtures, ECT and WCT, were slightly irritant, giving usually negative reactions and occasional ± reactions (in approximately 15 per cent of the animals). This primary irritation was due to tween 80 since ethanol and methyl cellosolve gave consistently negative

TABLE II
Results of Contact Sensitization of Guinea Pigs with Penicillin G and Its Degradation Products

Sensitizer*	No. of animals reacting				Percentage Sensitized†
	3+	2+	1+	0 and ±	
Potassium penicillin G	3	17	10	30	50
D-Penicillamine·HCl	5	24	7	5	88
D-Benzylpenicillenic acid	2	10	5	3	85
Monosodium-D-α-benzylpenicilloate	0	1	4	5	50
2-Benzyl-4-sodium hydroxymethylene-(5)-oxazolone	0	1	4	5	50
Benzylpenilloaldehyde	3	5	1	1	90

* Guinea pigs were sensitized by six percutaneous applications of 0.1 M solution of the sensitizer in a tween 80 solvent (see text).

† The animals were patch-tested for delayed contact hypersensitivity with a 0.1 M solution of the sensitizer in a tween 80 solvent. (See text.) Animals reacting 1+ or stronger were considered sensitized.

reactions. Under these experimental conditions, tween 80 was not a sensitizer. The materials tested, except for BPE, were not primary irritants at the concentrations used, giving reactions indistinguishable from the solvent reaction in non-sensitized animals. BPE at 0.1 M concentration was slightly more irritant than ECT solvent alone; five of ten animals gave ± reactions to 0.1 M BPE in ECT as compared to negative reactions for ECT alone. At concentrations of 0.01 M or less, BPE was non-irritant. Groups of four to six non-sensitized animals were used to test for primary irritation.

Only animals that had become sensitized were tested for cross-reactions. The solvent controls, tested simultaneously, were negative. Guinea pigs which reacted ± to the solvent were eliminated from tabulation. This introduced no bias, as these animals cross-reacted with the same patterns as did the others. It is to be noted that the slight solvent reactions might mask a faint allergic response, and conversely an occasional faint inflammatory response to a given test material may be due to a solvent reaction.

The Effect of Tween 80 Solvents on Sensitization and Elicitation.—The use of tween 80 mixtures as solvents for the test compounds was essential to effect both

sensitization and elicitation of the delayed contact allergic reactions. Tween 80, a non-ionic detergent, acts presumably by increasing intraepidermal penetration of these water-soluble allergens (4, 24). In PG-sensitized animals as well as in animals sensitized to both PSH and benzylpenilloaldehyde (Table III), testing with the sensitizer in ECT solution yielded much stronger allergic reactions than did testing with the sensitizer dissolved in a solvent mixture of 95 per cent

TABLE III
The Effect of Tween 80 Solvent on the Intensity of the Delayed Contact Allergic Reaction*

Elicitor†	Sensitized to benzylpenilloaldehyde (Pald)			
	Guinea pig No.			
	1	2	3	4
0.1 M Pald in ECT solvent	3+	2+	1+	1+
0.1 M Pald in EC Solvent	±	0	0	0
ECT solvent	0	±	0	0
EC solvent	0	0	0	0
Elicitor‡	Sensitized to D-penicillamine (PSH)			
	Guinea pig No.			
	1	2	3	4
0.1 M PSH in ECT solvent	3+	3+	1+	2+
0.1 M PSH in EC solvent	0	0	0	0
ECT solvent	0	±	0	0
EC solvent	0	0	0	0

* See text for methods of sensitization and elicitation.

‡ ECT solvent = 95 per cent ethanol; methylcellosolve; tween 80 (45:45:10 by volume). EC solvent = 95 per cent ethanol: methylcellosolve (1:1 by volume). The four elicitations were done simultaneously. PSH and Pald at 0.1 M concentration in ECT solution were not primary irritants.

ethanol and methylcellosolve (1:1 by volume) (EC). This same effect of tween 80 solvents has been noted by Eisen *et al.* (4) for 2,4-dinitrophenylsulfonate. Also, sodium dodecyl sulfate, an anionic detergent, in 10 per cent aqueous solution exhibited the same effect as tween 80 in markedly increasing the intensity of PSH-elicited allergic reactions in PSH-sensitized guinea pigs (25). However, this solvent caused the rapid degradation of PG, presumably to BPO (25).

Validation of the Use of Tween 80 Solvents.—The validity of the use of tween 80 solvents in investigating the allergic cross-reactivities among PG and its degradation products rests on the observations that tween 80 does not cause an artifactual degradation of PG, and that it does not destroy the specificity of the allergic response.

A 0.1 M solution of KPG dissolved in a 10 per cent tween 80 aqueous solution was found to develop an ultraviolet absorption peak at λ_{\max} 322 m μ (due to formation of BPE) at a much slower rate than did a 0.1 M solution of KPG in water alone. After standing at 25°C. for 24 hours, 0.050 ml. of the KPG aqueous tween 80 solution (5.0 micromols of KPG) was analyzed for PSH by paper chromatography. No evidence of PSH was found by the method described above, which is capable of detecting 0.5 molar per cent PSH in KPG. Thus, tween 80 did not increase the rate of formation of BPE from PG, nor did it cause the formation of PSH from PG.

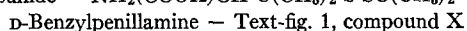
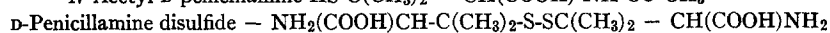
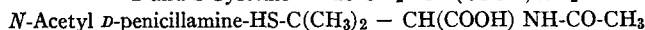
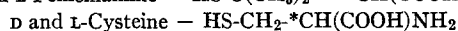
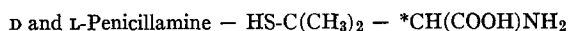
TABLE IV

*The Specificity of the Delayed Contact Allergic Reaction Using Tween 80 Solvents for Sensitization and Elicitation; Allergic Cross-Reaction to D-Penicillamine**

Elicitor†	Sensitized to D-penicillamine					
	Guinea pig No.					
	1	2	3	4	5	6
D-Penicillamine.....	2+	2+	2+	3+	3+	2+
L-Penicillamine.....	0	±	0	1+	±	0
D-Cysteine.....	0	0	0	±	±	0
L-Cysteine.....	0	0	0	0	0	0
N-Acetyl D-penicillamine.....	0	0	0	0		
D-Benzylpenillamine.....	0	0	0	0		
D-Penicillamine disulfide.....	0	0	0	0	0	0

* See text for methods of sensitization and elicitation. The five or seven tests plus a solvent control were done simultaneously.

† The elicitors were at 0.1 M concentration in ethanol; methyl cellosolve; tween 80 solution except for penicillamine disulfide which was at 0.08 M concentration in water-tween 80 solution (90:10 by volume). Solvent controls were 0. The elicitor solutions were not primary irritants.



Specificity of the Allergic Response to Elicitor in Tween 80 Solvent.—PSH-sensitized guinea pigs were tested with thiol compounds with varying structural differences from PSH (Table IV). The allergenic capacity of PSH probably depends on its ability to undergo a thiol-disulfide exchange reaction with cystine disulfide linkages of epidermal proteins to form D-penicillamine-cysteine mixed disulfide (PSSCy) residues (14, 26). Since low molecular weight thiol compounds are potent sensitizers (26), and thiol-disulfide exchange reactions have been demonstrated to occur in numerous instances (14, 26–28), it is probable that the other thiol compounds tested can also introduce mixed disulfide residues into epidermal proteins. As is clear from Table IV, PSH-sensitized animals reacted specifically to PSH, gave occasional faint cross-reactions to D-cysteine

and to L-penicillamine (L-PSH), and did not cross-react to L-cysteine, *N*-acetyl-D-penicillamine, D-benzylpenicillamine, or D-penicillamine disulfide. The faint cross-reactions to L-PSH may be due to racemization of L-PSH *in vivo*. Compounds such as penicillamine do undergo racemization readily (29). The formation of as little as 1 per cent D-PSH by racemization of L-PSH could account for these faint cross-reactions (*cf.* Table V).

Allergenic Equivalence.—The concept of allergenic equivalence is introduced to help determine the chemical pathways which are involved in the formation of the penicillin antigen by irreversible reaction of PG with epidermal proteins. By allergenic equivalence is meant the following: If two compounds, A and B, both introduce the same antigenic determinant groups into epidermal proteins, then if one group of guinea pigs is sensitized (by contact) with A, and another group is sensitized with B, the two groups will react indistinguishably when tested simultaneously with A and B. That is, the *ratio* of the intensities of the delayed contact allergic reactions to A and B will be the same for both groups, despite possible differences between the compounds in intraepidermal penetration or in chemical reactivity. When this pattern of allergic cross-reactivity is observed, the two compounds are said to be *allergically equivalent*. If two compounds are allergically equivalent, they can be considered to introduce the same antigenic determinant groups into epidermal proteins. If two compounds cross-react, but are not allergically equivalent, the compounds may introduce non-identical but structurally similar determinant groups into epidermal proteins, or one of these compounds must introduce into epidermal proteins at least one more antigenic determinant group than the other. It must be noted that since the method of grading intensity of contact allergic reactions is relatively gross, the assessment of allergenic equivalence is subject to some experimental error. Thus, if PG and one of its degradation products (compound X) are allergically equivalent, they must both form the same determinant groups by reaction with epidermal proteins. There are three possible chemical mechanisms which may account for this: (a) Both PG and X may react with proteins to form identical determinant groups. (b) Both PG and X may be further degraded *in vivo* to form a common proantigen.¹ (c) PG itself may be only negligibly reactive with proteins, but may degrade to X *in vivo*. Compound X and/or a further degradation product of X may be the proantigen(s) responsible for the formation of the penicillin antigen. These alternative mechanisms are considered in the Discussion.

Allergic Cross-Reactivity between Penicillin G (PG) and D-Penicillamine (PSH).—PSH was found to elicit allergic reactions in twelve of twenty guinea pigs, sensitized with PG. These reactions were usually weaker than were those with PG. Also, thirteen of twenty PSH-sensitized animals gave weaker cross-reactions to PG. Table V shows that PG and PSH were not allergically equivalent. PG-sensitized animals (group I) reacted more strongly to PG, whereas PSH-sensitized animals (group VI) reacted more strongly to PSH.

TABLE V
*Delayed Contact Allergic Cross-reactions among Potassium Penicillin G (KPG), D-Benzylpenicillic Acid (BPE), and D-Penicillamine (PSH), and Their Allergenic Equivalences**

Com- pound [§]	Con- cen- tra- tion mols/ liter	Sensitized with†											
		KPG			BPE			PSH					
		Group I	Group II	Group III	Group IV	Group V	Group VI						
KPG	0.1	2+ 2+ 3+ 2+ 2+ 2+	2+ 2+ 2+ 2+ 2+	2+ 2+ 2+ 2+ 2+	2+ 3+ 2+ 2+ 2+	2+ 2+ 2+ 2+ 2+	1+ 2+ 2+ 2+ 2+						
	0.01	1+ 1+ 1+ 1+ 2+	± 0 1+ ± 1+ ± 1+	1+ ± 1+ ± 1+ ± 1+	2+ 3+ 1+ 1+ 2+	0 0 0 0 0	1+ 1+ 2+ 0 ± 1+						
	0.001	0 0 0 0 0	0 0 0 ± 0 0	0 0 0 0 0	0 2+ 0 0 1+	0 0 0 0 0	± ± 1+ 0 0 1+						
BPE	0.1	2+ 2+ 2+ 1+ 2+	1+ 2+ 2+ 1+ 2+	2+ 2+ 2+ 2+ 2+	2+ 2+ 3+ 2+ 2+	2+ 2+ 2+ 2+ 2+	2+ 1+ ± 1+ ±						
	0.01	1+ 1+ 1+ 1+ 1+	1+ 1+ 1+ 1+ 1+	1+ 1+ 1+ 1+ ±	2+ 3+ 1+ 1+ 2+	0 0 0 0 0	0 0 0 0 0						
	0.001	± 0 0 ± 0 ± 0	± 0 ± 0 ± 0 ± 0	± ± ± ± ±	0 2+ 0 0 0	0 0 0 0 0	0 0 0 0 0						
PSH	0.1	2+ 2+ 1+ 0 1+ 0			± 2+ 1+ 1+ ±	3+ 2+ 2+ 2+ 2+	2+ 2+ 3+ 1+ 2+						
	0.01	1+ ± 0 0 0 0			0 1+ 0 0 0	2+ 1+ 1+ ± ±	2+ 2+ 2+ 1+ 1+						
	0.001	± 0 0 0 0 0			0 0 0 0 0	1+ 0 0 0 0	1+ 2+ 1+ ± ±						

* This table represents three separate experiments in which groups II and III were elicited simultaneously, as were groups IV and V and also groups I and VI.
† See text for methods of sensitization and elicitation. Each vertical column represents an individual guinea pig in which the six tests plus a solvent control were done simultaneously.
‡ The elicitors were dissolved in ethanol-methylcellosolve-tween 80 solvent. Solvent control reactions were 0. The elicitor solutions were not primary irritants except for 0.1 M BPE which gave ± reactions in 50 per cent of non-sensitized animals.

Allergic Cross-Reactivity between PG and D-Benzylpenicillenic Acid (BPE).—BPE was found to elicit strong allergic reactions in all six PG-sensitized guinea pigs and conversely all six BPE-sensitized animals reacted strongly to PG. Table V shows that BPE and PG were allergenically equivalent. Both PG-sensitized animals (group II) and BPE-sensitized animals (group III) gave equally intense, or slightly stronger reactions to BPE (Fig. 1).

TABLE VI
*Delayed Contact Allergic Reactivity of Various Degradation Products of Penicillin G in Penicillin G and D-Penicillamine Sensitized Guinea Pigs**

Elicited with †		Sensitized with ‡													
Compound §	Concentration	Penicillin G						D-Penicillamine							
	<i>mols/liter</i>														
Potassium penicillin G	0.1	2+	2+	2+	2+	1+	2+	2+	1+	2+	1+	1+	0	2+	0
D-Penicillamine	0.1	1+	1+	1+	±	0	1+	0	2+	2+	2+	2+	1+	3+	2+
D-Benzylpenillic acid	0.1	±	±	±	0	0			0	0	0	0	0		
D-Benzylpenillamine	0.1	±	0	0	0	0			0	0	0	0	0		
Benzylpenilloaldehyde	0.1	0	0	0	0	0	0	0							
Benzylpenilloaldehyde	0.5	0	0	0	0	0	0	0							
2-Benzyl-4-sodium hydroxymethylene-(5)-oxazolone	0.1	0	0	0	0	0	0	0							
2-Benzyl-4-sodium hydroxymethylene-(5)-oxazolone	0.5	0	0	0	0	0	0	0							
Monosodium D-α-benzylpenicilloate	0.1	±	1+	±	±		0	±	1+	2+	0	1+	1+	2+	2+

* This table represents a composite of four separate experiments. In the vertical groups, the reaction to the degradation product is compared with the reactions to penicillin G and to D-penicillamine; these three or four tests and a solvent control were done simultaneously on an individual animal.

† See text for methods of sensitization and elicitation.

§ The test compounds were dissolved in ethanol-methylcellosolve-tween 80 (ECT) solvent. D-Benzylpenillic acid required 0.9 ml. ECT + 0.1 ml. of 1 N NaOH to effect solution, and monosodium D-α-benzylpenicilloate was dissolved in water-methylcellosolve-tween 80 solvent. Solvent control reactions were 0. The test solutions were not primary irritants.

Allergic Cross-Reactivity between PSH and BPE.—As seen in Table V and Fig. 1, PSH and BPE cross-reacted in six of ten animals, but the two compounds were not allergenically equivalent. BPE-sensitized animals (group IV) reacted more strongly to BPE, and PSH-sensitized animals (group V) reacted more strongly to PSH.

Allergic Reactivity of D-Benzylpenicilloic Acid (BPO).—BPO elicited weak allergic reactions in PG-sensitized guinea pigs and relatively stronger reactions in PSH-sensitized animals (Table VI). BPO and PSH were not allergenically equivalent (Table VII), nor were BPO and PG allergenically equivalent (Tables VI and VII). Benzylpenilloaldehyde, another degradation product of BPO, gave consistently negative reactions in BPO-sensitized animals (Table VII).

TABLE VII

*Delayed Contact Allergic Reactivity of D-Benzylpenicilloic Acid, D-Penicillamine, Penicillin G, and Benzylpenilloaldehyde in Guinea Pigs Sensitized with D-Penicillamine and D-Benzylpenicilloic Acid; Allergic Inequivalence between D-Benzylpenicilloic Acid and D-Penicillamine**

Elicited with‡		Sensitized with‡										
Compound§	Concentration	Monosodium D- α -benzylpenicilloate					D-Penicillamine					
	<i>mols/liter</i>											
Monosodium D- α -benzylpenicilloate	0.1	2+	1+	1+	1+	1+	1+	1+	0	2+	1+	1+
D-Penicillamine	0.1	2+	1+	1+	1+	±	2+	2+	2+	3+	2+	2+
Potassium penicillin G	0.1	1+	1+	1+	±	0	1+					
Benzylpenilloaldehyde	0.1	0	0	±	0	0	0	0	0	0	0	0
Benzylpenilloaldehyde	0.5	0	0	0	0	0	0	0	0	0	0	0

* Each vertical column represents a single guinea pig tested simultaneously with the four or five test compounds and two solvent controls.

‡ See text for methods of sensitization and elicitation.

§ The text compounds were dissolved in ethanol-methylcellosolve-tween 80 solvent except for monosodium D- α -benzylpenicilloate which was dissolved in water-methylcellosolve-tween 80. Solvent reactions were 0. The elicitor solutions were non-irritant.

TABLE VIII

*Delayed Allergic Contact Reactivity of Penicillin G in Guinea Pigs Sensitized with Benzylpenilloaldehyde and with 2-Benzyl-4-Sodium Hydroxymethylene-(5)-Oxazolone (BSO); Allergic Cross-Reactions between Benzylpenilloaldehyde and BSO**

Elicited with‡		Sensitized with‡									
Compound§	Concentration	Benzylpenilloaldehyde					BSO				
	<i>mols/liter</i>										
Benzylpenilloaldehyde	0.1	2+	2+	2+	2+	3+	±	0	0	0	1+
BSO	0.1	1+	2+	1+	1+	1+	1+	1+	1+	1+	2+
Potassium penicillin G	0.1	0	0	0	0	0	0	0	0	0	0
Potassium penicillin G	0.5	0	0	0	0	0	0	0	0	0	0

* Each vertical column represents an individual guinea pig elicited simultaneously with the four test solutions and a solvent control.

‡ See text for methods of sensitization and elicitation.

§ The compounds were dissolved in ethanol-methylcellosolve-tween 80 (ECT) solvent except for 0.5 M potassium penicillin G, which required 0.9 ml. ECT + 0.1 ml. H₂O to effect solution. Solvent control reactions were 0. The test solutions were not primary irritants.

Allergic Reactivity of D-Benzylpenillic Acid and D-Benzylpenillamine.—D-Benzylpenillic acid gave occasional faint reactions in PG-sensitized animals and negative reactions in PSH-sensitized animals (Table VI). D-Benzylpenillamine gave negative reactions in both groups (Table VI).

Allergic Reactivity of Benzylpenilloaldehyde and 2-Benzyl-4-Sodium Hydroxy-

methylene-(5)-Oxazolone (BSO).—Both compounds gave consistently negative reactions in PG-sensitized animals (Table VI), and conversely, PG gave negative reactions in both BSO- and benzylpenilloaldehyde-sensitized animals (Table VIII). Benzylpenilloaldehyde and BSO cross-reacted with each other (Table VIII).

Allergic Reactivity of Penicillin O in PG- and PSH-Sensitized Guinea Pigs.—Penicillin O elicited allergic cross-reactions in both PG- and PSH-sensitized animals (Table IX). Two PG-sensitized animals which cross-reacted to penicillin O, gave negative reactions to PSH (Table IX).

TABLE IX
Delayed Contact Allergic Reactivity of Sodium Penicillin O (NaPO) in Guinea Pigs Sensitized with Potassium Penicillin G (KPG) and with D-Penicillamine (PSH)

Elicited with ‡		Sensitized With*											
Compound	Concentration	KPG					PSH						
	<i>mols/liter</i>												
KPG	0.1	2+	2+	3+	2+	2+	3+	1+	2+	2+	±	±	2+
NaPO	0.1	2+	2+	2+	1+	2+	2+	1+	2+	1+	±	±	2+
PSH	0.1	2+	2+	1+	0	1+	0	2+	2+	3+	1+	2+	3+

* See text for methods of sensitization and elicitation. Each vertical column represents an individual guinea pig in which the three tests and a solvent control were done simultaneously.

‡ The elicitors were dissolved in ethanol-methylcellosolve-tween 80 solvents. Solvent controls were 0. The test solutions were not primary irritants.

Summary of the Allergic Reactivity of the Degradation Products of PG.—Text-fig. 1 summarizes the allergic reactivity of the degradation products of PG. In parentheses, following the name of the compound, is placed the average intensity of the allergic reaction given by a 0.1 M ECT solution of the compound (BPO is dissolved in WCT) in a PG-sensitized guinea pig reacting 2+ to 0.1 M PG in ECT solution.

DISCUSSION

The foregoing experimental results describe the allergic cross-reactivities, which exist among penicillin G (PG) and its degradation products, using guinea pig allergic contact dermatitis as the test system. PG and D-benzylpenicillenic acid (BPE) were found to be allergenically equivalent (Table V) (Fig. 1) (see Experimental section for definition of allergenic equivalence). One possible explanation for the observed allergenic equivalence between PG and BPE is that both PG and BPE may react irreversibly with epidermal proteins to form the same antigenic determinant groups. However, PG has not been demonstrated to react irreversibly with proteins, or with "protein model"

compounds such as alanine, cystine, or *N*-acetyl cysteine in neutral aqueous solution (9, 10). Although PG does react with β -aminothiols such as cysteine (9, 10), *N*-terminal cysteine has not been found to occur in many proteins examined (30–33).

Another possible explanation for the allergenic equivalence between PG and BPE is that PG may rearrange *in vivo* to BPE which then reacts irreversibly with proteins to form the penicillin antigen. There is considerable evidence in support of this view: Firstly, BPE is a highly reactive compound. In pH 7.5 phosphate buffer solution at 25°, BPE reacts rapidly with the NH₂ group of β -alanine to yield a mixture of diastereomers of β -alanyl- α -amide of BPO^{10a} (13). Under these conditions BPE appears to react rapidly with the disulfide linkage of oxidized glutathione to yield BPE-glutathione mixed disulfide (13). Experiments now in progress (25) indicate that BPE reacts also with lysine ϵ -NH₂ groups and to a lesser extent with cystine disulfide linkages¹¹ of both human γ -globulin and human plasma albumin at pH 7.5. Based on these reactions under physiological conditions *in vitro*, it may be anticipated that BPE might react with epidermal proteins *in vivo*. Secondly, it appears possible that PG may rearrange to BPE at a fairly rapid rate *in vivo*. In pH 7.5 phosphate buffer solution at 37°, PG is hydrolyzed as a first order reaction at a rate of 1.2 per cent per hour (17). The reaction products are mainly a mixture of diastereomers of BPO, but the appearance of a small quantity of BPE was detected in the degrading PG solution (12). Under identical conditions, BPE is hydrolyzed to yield a mixture of diastereomers of BPO at a rate of 11 per cent per minute (13). Since BPE is hydrolyzed 550 times more rapidly than PG, the appearance of a small amount of BPE in the degrading PG solution indicates that PG is hydrolyzed at least partially through the intermediate BPE. In human and rabbit serum solution at pH 7.5 and 37°, PG degrades at a rate of 10 per cent per hour (34), presumably to BPO. This marked increase in the rate of degradation of PG effected by serum may be due to an increase in the rate of rearrangement of PG to BPE. Eagle (34) has demonstrated that the responsible serum factors are thermostable. Since trace quantities of metal ions such as Fe⁺⁺ and Cu⁺⁺ markedly increase the rate of hydrolysis of PG to BPO in aqueous solution *in vitro* (18 e, 19 k), it is possible that traces of such metal ions present in serum may be responsible for the rapid degradation of PG in serum solution. Chain (18 e) has suggested that the effect of these metal ions is on the sulfur atom leading to the rupture of the thiazolidine ring and the formation of an unstable intermediate, which we believe to be BPE, that is rapidly hydrolyzed to BPO. Thus, it is possible that PG may rearrange to BPE at a rate in the order of 10 per cent per hour *in vivo*.

The data presented above suggest that PG may react with tissue proteins *in vivo* primarily through the active intermediate BPE. These data however, do not exclude the possibility that PG itself may also react with proteins to some extent.

^{10a} β -alanyl- α -amide of BPO is more correctly termed *N*-(*D*- α -benzylpenicilloyl)- β -alanine. Similarly, ϵ -lysyl- α -amide of BPO is more correctly termed ϵ -*N*-(*D*- α -benzylpenicilloyl) lysine.

¹¹ The reaction of BPE with disulfide linkages of these proteins was carried out in the presence of 0.1 M sodium decyl sulfate.

It might be anticipated that if BPE is the proantigen responsible for the *in vivo* formation of the penicillin antigen, then BPE should give strikingly stronger allergic reactions than does PG. In Table V it can be seen that BPE gave allergic reactions of equal or only slightly greater intensity than did PG in both PG- and BPE-sensitized animals. This observation, however, is not inconsistent with the view that PG reacts irreversibly with epidermal proteins through the reactive intermediate, BPE. BPE would probably react with keratin in the outer, cornified epidermal layer. Thus, the concentration of BPE reaching the sites in the basal epidermal layers necessary to elicit the allergic reaction (35) would be less than the concentration of PG. Furthermore, since BPE is hydrolyzed 550 times more rapidly than PG under physiological conditions *in vitro*, the concentration of BPE in epidermis would be considerably less than that of PG after a short period of time; *e.g.*, 1 hour. In this regard, although 2,4-dinitrofluorobenzene reacts 200 times more rapidly with aniline, and presumably with free amino groups of proteins than does 2,4-dinitrochlorobenzene (36), the two compounds give allergic reactions of equal intensity in guinea pigs with allergic contact dermatitis to 2,4-dinitrofluorobenzene (37).

One of four commercial PG preparations assayed for BPE impurity contained a maximum of 0.18 per cent BPE.¹² The presence of this small amount of BPE impurity may not be greatly significant if PG rearranges to BPE at a fairly rapid rate *in vivo*. However, the presence of free BPE in commercial PG preparations may be responsible, in part, for allergic reactions to PG which occur within seconds after its administration (38).

The allergic cross-reactivity between PSH and both PG and BPE (Table V) merits discussion. The allergenic activity of PSH (Table II) is probably due to its ability to undergo a thiol-disulfide exchange reaction with cystine disulfide linkages of epidermal proteins to form PSSCy residues (14, 26). Its cross-reactions with PG and BPE would appear to be due to the ability of both PG and BPE to introduce PSSCy residues into epidermal proteins. The alternative possibility that PSH cross-reacts with PG and BPE because of the structural similarity between PSSCy residues and either BPE-cysteine residues or ϵ -lysyl- α -amide of BPO residues is unlikely (see Text-fig. 2). This statement is based on the lack of contact allergic cross-reactivity to PSH exhibited by thiol compounds with structural similarity to PSH (Table IV).

PG and BPE appear to introduce PSSCy groups into epidermal proteins by being first hydrolyzed to BPO which reacts with cystine disulfide linkages to form PSSCy

¹² Three commercial preparations of potassium penicillin G (Pfizer, lot 72041-34100; Lilly, lot 7013-698920; and Bristol, lot E-7941) contained no detectable BPE. One commercial preparation of sodium penicillin G (Pfizer, lot 74-495) contained 0.18 per cent BPE. This preparation gave a faintly positive nitroprusside test whereas the potassium penicillin G preparations were negative. BPE assays were done spectrophotometrically as described in the Experimental section.

groups. Supporting this view are: (a) BPO does sensitize guinea pigs to PSH (Table VII). (b) BPO¹³ reacts with cystine in pH 7.5 aqueous solution to yield PSSCy (15). PSSCy residues may be formed also by pathways other than the one proposed above, but as yet there is no clear experimental evidence in support of other pathways.¹⁴ This proposed mechanism for the formation of PSSCy residues offers an explanation for the lack of allergic cross-reactivity between benzylpenilloaldehyde or BSO and PG (Tables VI and VIII). With scission of the postulated intermediate, D-benzylpenamaldate-cysteine,¹³ one mol of benzylpenilloaldehyde¹⁵ would be liberated free in tissue for every mol of PSSCy already bound in proteins. Only a small percentage of the free benzylpenilloaldehyde would be expected to react with epidermal proteins (35) presumably because of the competitive diffusion of benzylpenilloaldehyde out of the epidermis and possibly also because of its degradation in epidermis (*e.g.*, by oxidation).

Lastly, since BPO and PSH were not allergenically equivalent, BPO must introduce at least one antigenic determinant group other than PSSCy into epidermal proteins. The other determinant group may be D-benzylpenamaldate-cysteine mixed disulfide (see footnotes 13 and 15).

Based on the immunological and chemical considerations presented above, the schema in Text-fig. 2 is proposed as a possible mechanism whereby PG may react with epidermal proteins *in vivo* to form the penicillin antigen. It is proposed that PG rearranges to BPE; BPE reacts with lysine ϵ -NH₂ groups and cystine disulfide linkages of epidermal proteins to form a mixture of the diastereomers of ϵ -lysyl- α -amide of BPO,^{10a} and BPE-cysteine residues re-

¹³ The reactive form of BPO is believed to be D-benzylpenamaldic acid (VI), traces of which appear to exist in tautomeric equilibrium with BPO at pH 7.5. In support of this view is: Treatment of a D- α -benzylpenicilloate solution at pH 7.5 with mercuric chloride caused the immediate formation of an intense ultraviolet absorption peak at λ 280 m μ . This peak at λ 280 m μ has been shown to be due to D-benzylpenamaldic acid (19 a). Also, the mutarotation observed for D- α -benzylpenicilloate in pH 7.5 buffer solution (12) probably proceeds through the reversible formation of D-benzylpenamaldic acid which would cause epimerization of two of the three asymmetric carbon centers of BPO. The BPO-cystine reaction may proceed through the formation of D-benzylpenamaldic acid-cysteine mixed disulfide which is a Schiff base and would accordingly be expected to degrade to PSSCy (Text-fig. 2). Although this degradation is instantaneous in acid solution (18 b, 19 a), it may proceed slowly at pH 7.5.

¹⁴ PSSCy residues may be formed by degradation of BPE-cysteine residues *in vivo*. Evidence against this possibility is that both BPE-glutathione mixed disulfide and BPE disulfide appear to be stable in pH 7.5 buffer solution (13). The degradation of PG *in vivo* directly to PSH has not been clearly demonstrated (see footnote 3).

¹⁵ Actually, scission of D-benzylpenamaldate-cysteine would liberate benzylpenaldate ion (18 b, 19 a). Under acid conditions *in vitro*, benzylpenaldic acid is immediately decarboxylated to benzylpenilloaldehyde (18 c) (Text-fig. 1). This decarboxylation appears to occur also *in vivo*, since BSO and benzylpenilloaldehyde cross-react with each other (Table VIII). These cross-reactions are probably due to the *in vivo* degradation of BSO to benzylpenilloaldehyde which proceeds through the intermediate, benzylpenaldic acid (19 d). Since PG and BSO do not cross-react even at 0.5 M concentration (Tables VI, VIII), it appears unlikely that benzylpenaldic acid is a significant proantigen responsible for PG allergy.

spectively. BPE is also hydrolyzed to BPO; BPO rearranges to D-benzylpenamaldic acid which reacts with cystine disulfide linkages to yield D-benzylpenamaldate-cysteine residues. PSSCy residues are considered to be formed mainly by degradation of D-benzylpenamaldate-cysteine and possibly also by degradation of BPE-cysteine residues.¹⁴ The antigenic determinants responsible for PG allergic contact dermatitis in the guinea pig would then be: (a) the diastereomers of ϵ -lysyl- α -amide of BPO, (b) BPE-cysteine mixed disulfide, (c) D-benzylpenamaldate-cysteine mixed disulfide, and (d) PSSCy. It must be stressed that other possible pathways for the formation of the penicillin antigen cannot be excluded, e.g. the direct reaction of PG with epidermal proteins, and that the identity of the antigenic determinant groups has not been directly demonstrated, but is based on model considerations.

The cross-reactions noted to occur among penicillins G and O and PSH (Table IX) indicate that the two penicillins can cross-react by virtue of the common formation of the PSSCy determinant group. Since two PG-sensitized animals cross-reacted to penicillin O but not to PSH (Table IX), the two penicillins may cross-react also because of the structural similarity between the benzyl and allylmercaptomethyl homologues of the other three antigenic determinant groups (Text-figs. 1 and 2).

It may be that a similar chemical pathway is involved in the formation of the penicillin antigen responsible for PG allergy of the immediate type in man. In numerous instances, low molecular weight compounds which are allergenic for the guinea pig are allergenic for man (26, 39). Also, intracutaneous sensitization of animals with low molecular weight allergens has induced both delayed contact allergy and immediate, or serum antibody-dependent, allergy against the same simple antigenic determinant group (1, 2, 6, 37, 40-42). With regard to PG allergy, positive patch tests have been obtained to PSH in two of three patients with allergic contact dermatitis to PG (43), and also to BPE in the one such patient tested (25). Also, allergic reactions of the immediate type to D,L-penicillamine have been observed in several patients under treatment for Wilson's disease (44). However, it cannot be stated that the same determinant groups responsible for PG allergic contact dermatitis in the guinea pig are also responsible for PG allergy of the immediate type in man. Aside from the possibility that PG may be degraded differently in man, differences may arise from the nature of the proteins with which PG reacts. Epidermal proteins contain relatively large numbers of reactive cystine disulfide linkages (5). Accordingly, the mixed disulfide residues would be expected to be important determinant groups in PG allergic contact dermatitis. Indeed, at least one of the possible mixed disulfide determinant groups, PSSCy, has been clearly demonstrated to be an important specific determinant of PG allergic contact dermatitis. Approximately 60 per cent of PG-sensitized guinea pigs, and two of three patients with PG contact dermatitis gave positive allergic cross-reactions

to PSH. Serum albumins and globulins which may be important carrier proteins involved in the formation of the PG antigen responsible for the immediate type of allergy, are relatively poor in reactive cystine disulfide linkages (45), and contain large numbers of reactive free lysine ϵ -NH₂ groups. Thus, the more important determinant groups responsible for serum antibody-dependent allergy to PG may be the mixture of diastereomers of ϵ -lysyl- α -amide of BPO.¹⁶

Studies are currently in progress (47) to determine the identity of the antigenic determinant groups responsible for PG allergy of the immediate type in man. In early experiments, a soluble protein conjugate prepared by reacting BPE with human γ -globulin at pH 7.5, has been successfully employed as an antigen to detect antipenicillin antibody in the serum of human beings with PG allergy of the immediate type. This conjugated antigen appears to contain mainly the ϵ -lysyl- α -amide of BPO determinant groups.

The identification of the allergic determinant groups responsible for the immediate type of PG allergy in man may lead to the preparation of soluble conjugated protein antigens useful as clinical reagents for the diagnosis of PG allergy. Also, low molecular weight haptene inhibitors can be prepared which may have clinical application in the specific immunological prevention and treatment of PG allergy. Finally, since PG is used widely as a therapeutic agent, and allergic reactions to PG are common, the penicillin system may be useful as a model for the study of more general problems in human allergic disease.

SUMMARY

Seven highly purified degradation products of penicillin G (PG) were examined with regard to their ability to cross-react allergically with PG. Guinea pig allergic contact dermatitis was employed as the test system. Three of these degradation products, D-benzylpenicillenic acid (BPE), D-penicillamine, and D- α -benzylpenicilloic acid were found to cross-react with PG and also to be capable of inducing delayed contact allergy in the guinea pig. BPE and PG cross-reacted with particularly intense reactions, and other immunologic experiments indicated that PG and BPE introduce identical allergic determinant groups into epidermal proteins.

These experimental results were correlated with the results of previous studies concerning the degradation pathways of PG under physiological conditions *in vitro*, and the chemical reactivities of these degradation products.

Based on these immunologic and chemical data, a schema is proposed which suggests the chemical pathways by which PG may react with epidermal proteins

¹⁶ Consistent with this possibility are the observations of Josephson (46), who demonstrated that agglutination of "penicillin-coated" red blood cells by rabbit "antipenicillin" sera could be inhibited by D-benzylpenicilloic acid.

in vivo to form the penicillin antigen. The identity of the specific antigenic determinant groups of the penicillin antigen is suggested.

The relationship between PG allergy of the contact dermatitis type in the guinea pig and PG allergy of the immediate type in man is discussed.

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EXPLANATION OF PLATES

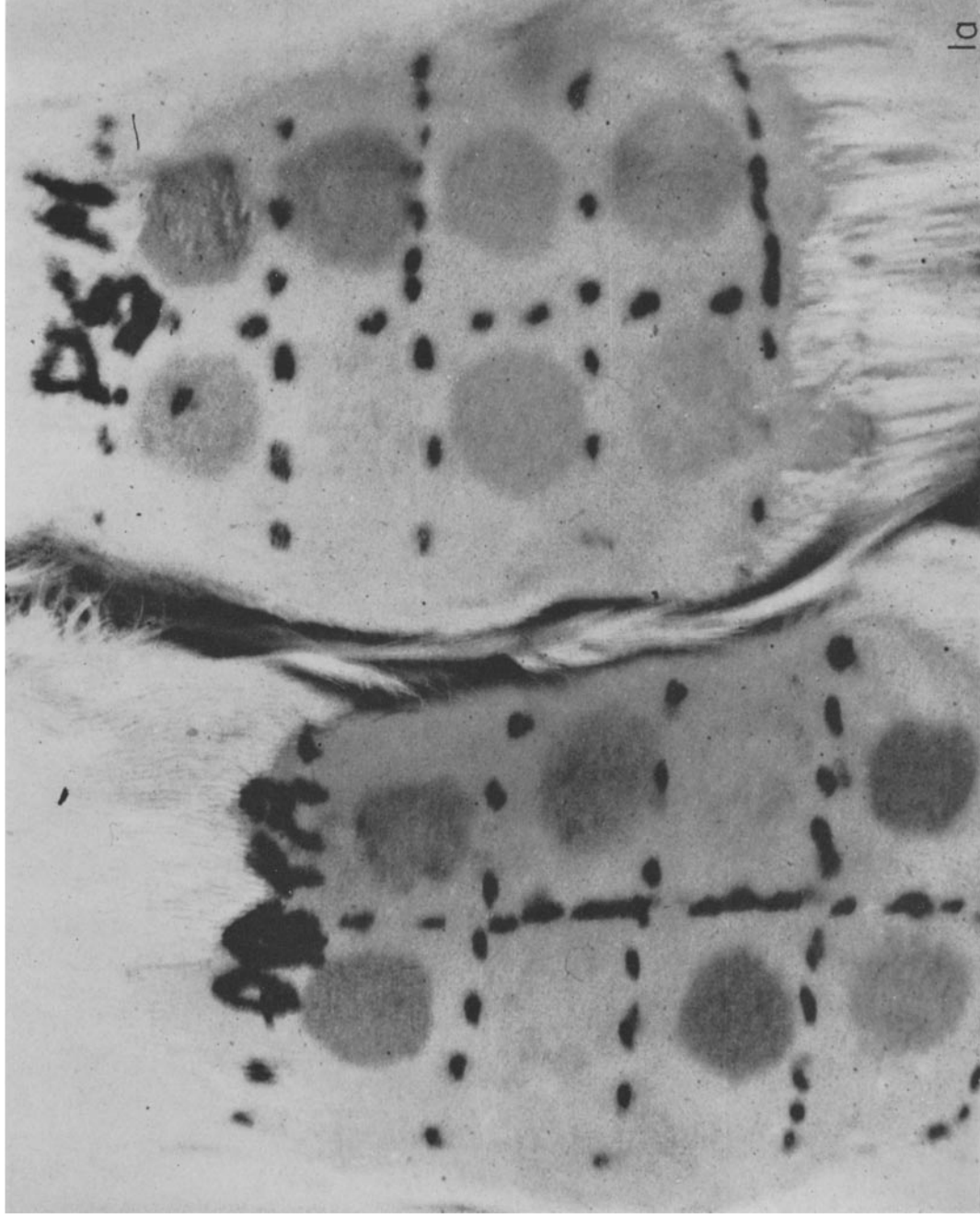
PLATE 94

FIG. 1 *a*. Allergenic inequivalence between D-benzylpenicillenic acid (DBPA) and D-penicillamine (PSH). One guinea pig sensitized with DBPA and another sensitized with PSH were tested with:

1	5
2	6
3	7
4	8

1. 0.1 M DBPA	5. 0.1 M PSH
2. Solvent control	6. 0.01 M PSH
3. 0.01 M DBPA	7. 0.001 M PSH
4. 0.001 M DBPA	8. 0.1 M penicillin G

The solvent was ethanol; methylcellosolve; tween 80 (45:45:10 by volume) (ECT). The DBPA-sensitized animal exhibited stronger reactions to DBPA, whereas the PSH-sensitized animal gave stronger reactions to PSH. Both animals cross-reacted to penicillin G.



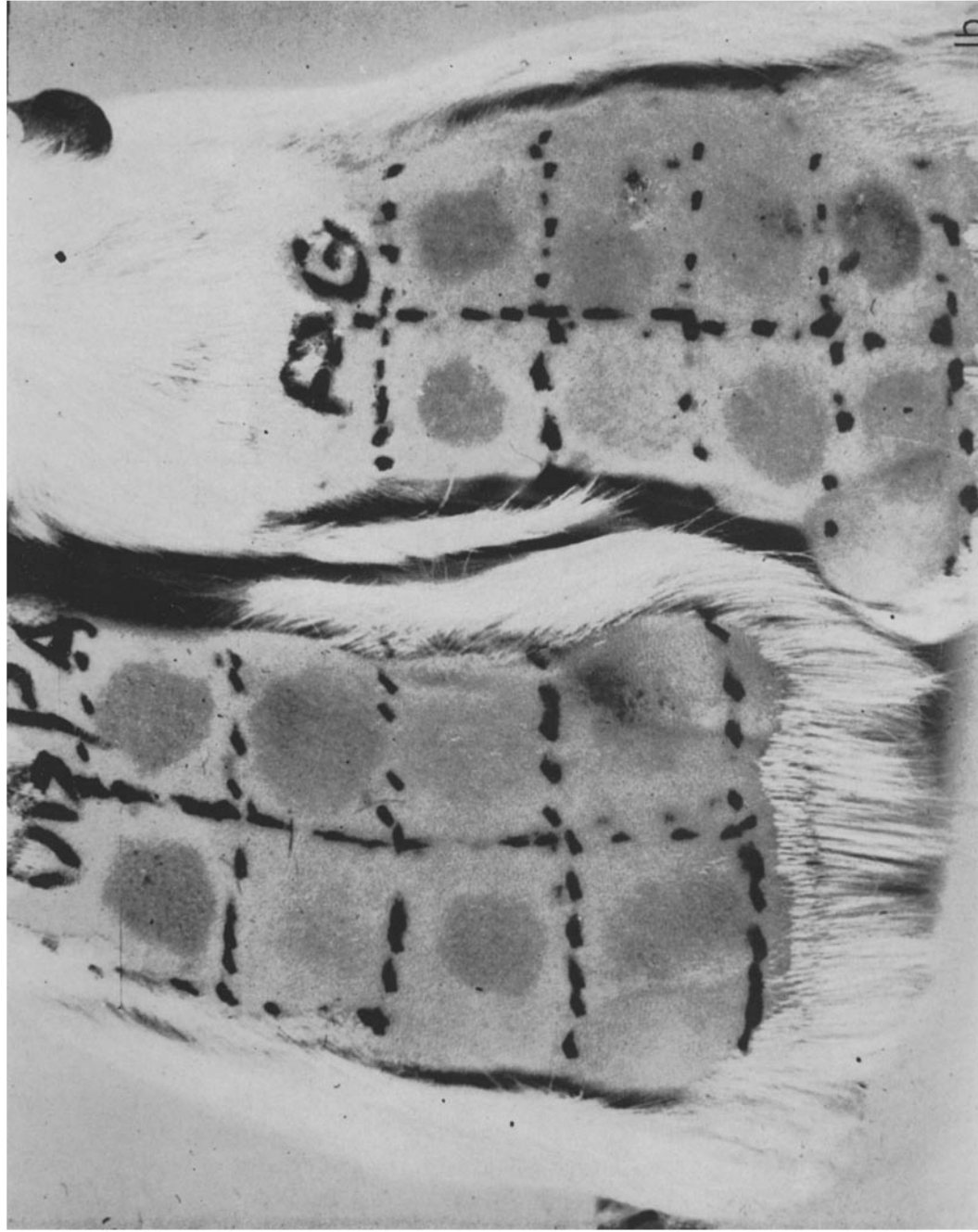
(Levine: Formation of penicillin antigen)

PLATE 95

FIG. 1 *b*. Allergenic equivalence between DBPA and penicillin G (PG). One guinea pig sensitized with DBPA and another sensitized with PG were tested with ECT solutions of:

1	5
2	6
3	7
4	8
	1. 0.1 M DBPA
	2. Solvent control
	3. 0.01 M DBPA
	4. 0.001 M DBPA
	5. 0.1 M PG
	6. 0.01 M PG
	7. 0.001 M PG
	8. 0.1 M PSH

Both the DBPA-sensitized animal and the PG-sensitized animal gave equally intense reactions to DBPA and PG at 0.1 M concentration. At 0.01 M and 0.001 M concentrations, both animals gave slightly stronger reactions to DBPA. Both animals cross-reacted to PSH.



(Levine: Formation of penicillin antigen)