STUDIES ON THE MECHANISM OF THE FORMATION OF THE PENICILLIN ANTIGEN

III. The N-(D-α-Benzylpenicilloyl) Group as an Antigenic Determinant Responsible for Hypersensitivity to Penicillin G*,‡

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Although penicillin G (PG)[1] has long been known to be a clinical allergen, the identity of the antigenic determinant groups responsible for hypersensitivity to PG is not clearly known. Such knowledge would be of importance since it would lead to the preparation of conjugated protein antigens useful as reagents to diagnose penicillin hypersensitivity. This information would make possible also the synthesis of low molecular weight univalent haptens which might be useful, therapeutically, to specifically inhibit antibody-dependent penicillin allergic reactions. Also, with the clear definition of the responsible antigenic determinant groups, the penicillin system may be employed as a model to study more general problems of human hypersensitivity.

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[1] The following abbreviations are used in this report:

PG, benzylpenicillin (G)

NaPG, KPG, Sodium or potassium PG

BPE, D-benzylpenicillenic acid

BPE-SS-Cys, cysteine mixed disulfide group

BPO, D-benzylpenicilloyl group

Di-BPO, diastereomeric mixture of BPO groups

BPO-EACA, N-(D-α-benzylpenicilloyl)-ε-aminocaproic acid

α-BPO-EACA, α-diastereomer of BPO-EACA

Di-BPO-EACA, diastereomeric mixture of BPO-EACA

BPO-Lys, ε-N-(D-α-benzylpenicilloyl)-lysine groups

α-BPO-Lys and Di-BPO-Lys, α-diastereomer and diastereomeric mixture of BPO-Lys

Penamaldoyl-EACA, ε-N-(D-α-benzylpenamaldoyl)-lysine group

D-penicillamine-SS, D-penicillamine mixed disulfide groups

B.S., Buffered saline (0.15 M NaCl + 0.02 M potassium hydrogen phosphate adjusted to pH 7.2.)

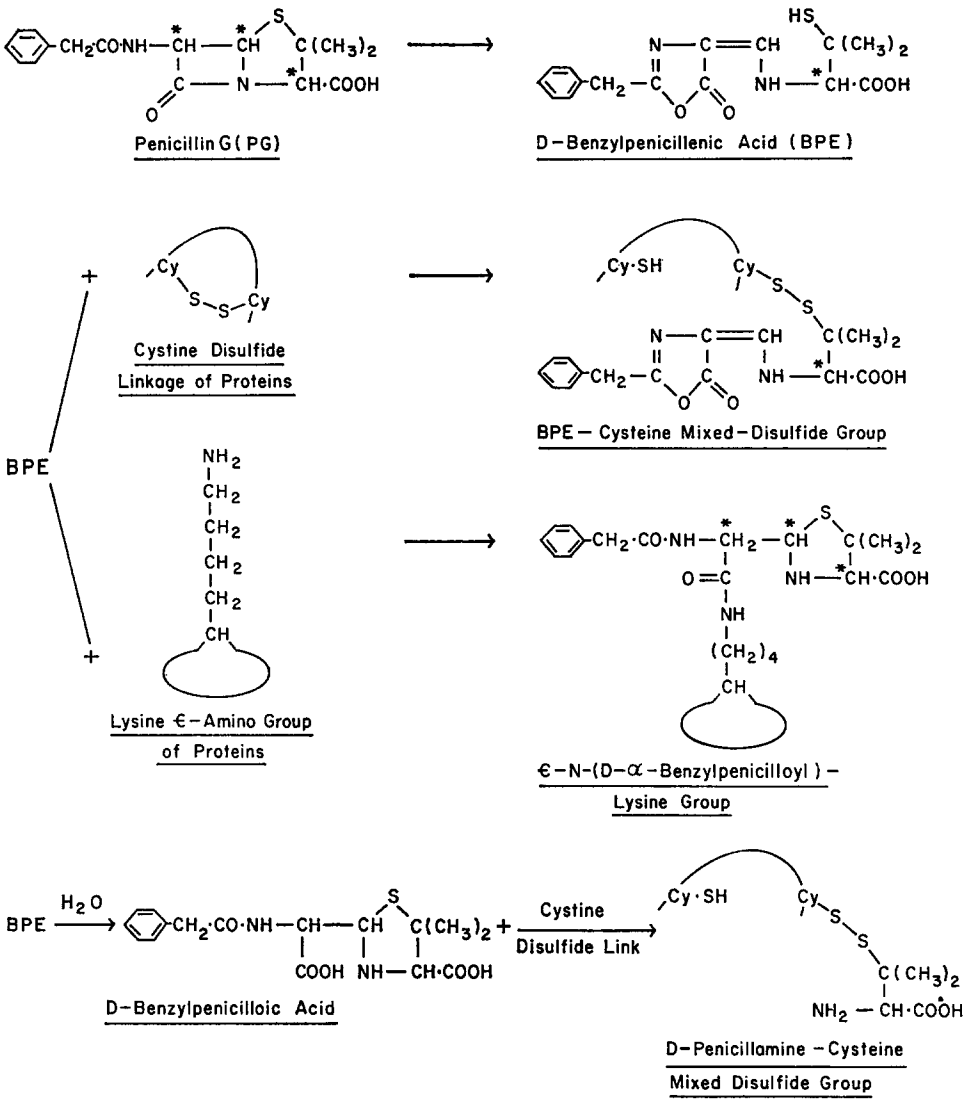
In previous studies on PG allergic contact dermatitis, it was suggested that PG might react with ϵ-NH_2 groups and cystine disulfide linkages of epidermal proteins through its rearrangement product D-benzylpenicillenic acid (BPE). The resulting derivative groups, the diastereomeric mixture of ϵ-N-(D-α-benzylpenicilloyl)-lysine (Di-BPO-Lys), BPE-cysteine mixed disulfide (BPE-SS-Cys) and D-penicillamine-cysteine mixed disulfide might be the major antigenic determinant groups responsible for PG hypersensitivity of the contact dermatitis type (1–4) (Text-fig. 1).

With regard to PG hypersensitivity of the immediate (or antibody-dependent) type, the consideration that reactive ϵ-amino groups are much more numerous in native serum proteins than are reactive cystine disulfide linkages (5), prompted the suggestion that the Di-BPO-Lys groups may be the more important antigenic determinants against which "anti-penicillin" antibodies are directed (1). Consistant with this possibility are the observations of Josephson (6), who demonstrated that the agglutination of "penicillin-coated" red blood cells by rabbit "anti-penicillin" sera could be inhibited by D-benzylpenicilloic acid. More recently de Weck and Eisen (7) demonstrated that BPE reacts with cystine disulfide linkages of native proteins and with thiol groups of thiolated proteins at pH 8.5 to 9.0, in the presence of 8 M urea and H_2O_2 to form conjugates containing BPE mixed disulfide groups (BPE-SS). They found that guinea pigs and rabbits immunized with these conjugates developed serum antibodies specific for the BPE-SS group and no antibodies directed against the Di-BPO group were detected. Based on these data, de Weck and Eisen suggested that the BPE-SS group may be an antigenic group responsible for PG hypersensitivity of the immediate type (7).

In the present work, the *in vitro* reactions between BPE and various proteins in aqueous solution at pH 7.5 to 8.0 were investigated. Rabbits were immunized with PG and the specificities of the resulting "anti-penicillin" antibodies were studied. It was found that the reaction between BPE and native human gamma globulin and human serum albumin, resulted in conjugates containing both Di-BPO-Lys groups and BPE-SS-Cys groups, but considerably more of the former than the latter. Rabbits immunized with PG developed serum antibodies directed against the Di-BPO groups and no antibodies directed against the BPE-SS and the D-penicillamine-SS groups could be detected. Several patients with histories of allergic urticarial reactions to PG were skin-tested and found to react specifically with wheal and erythema responses to an antigen containing Di-BPO-Lys groups. One patient under treatment with 25 gm KPG per day was found to have antigenic non-dialyzable BPO conjugates in his serum. Another patient with a recent allergic reaction to PG was found to have serum antibodies specific for the BPO group. It is concluded that Di-BPO groups (predominantly Di-BPO-Lys groups) are formed *in vivo* by reaction of PG with tissue proteins (presumably through the intermediate BPE) and that these groups are the major antigenic determinants responsible for hypersensitivity to PG.

EXPERIMENTAL AND RESULTS

Materials.—Human gamma globulin (HGG) (Fx II) (Squibb, batch 1874) was obtained through the courtesy of the American Red Cross. Human serum albumin (HSA) was obtained from the American Red Cross as a 25 per cent aqueous solution. It was dialyzed several

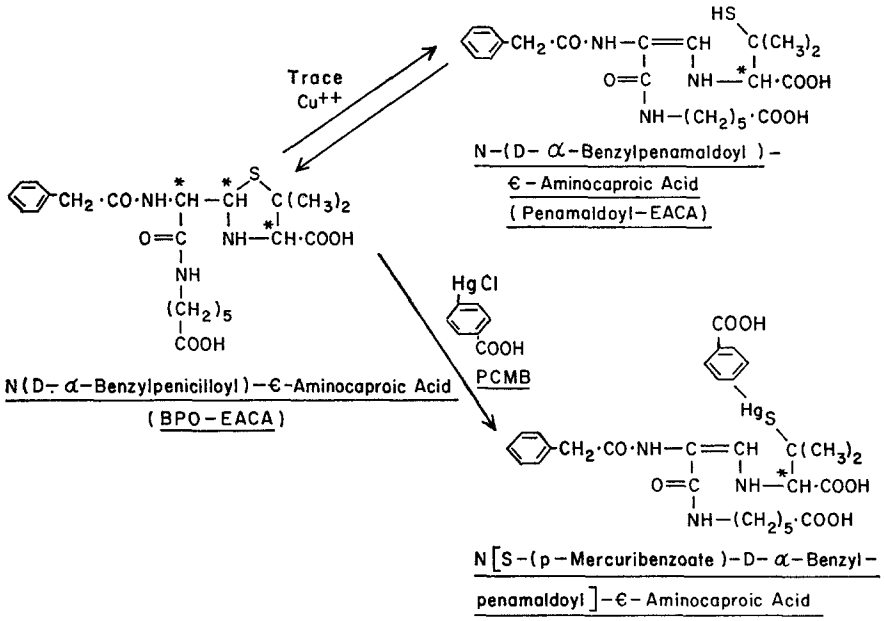


TEXT-FIG. 1. Postulated routes of formation of several possible penicillin antigenic determinant groups. (*) refers to an assymetric carbon center.

times against water and lyophilized. Purified calf-skin gelatin (Gel) was a product of Eastman Chemicals Co., Rochester, New York. Poly-l-lysine (PL) (molecular weight = 180,000) was obtained as the hydrochloride from Mann Laboratories, New York. Protein contents of these preparations were assayed by micro-Kjeldahl analysis (8 *a*).

Crystalline NaPG (1650 units per mg) was furnished by Pfizer Laboratories, Brooklyn. BPE was prepared by rearrangement of NaPG as described previously, (2, 9). Crystalline

KPG (1595 units per mg), Lot C-0611, was furnished by Bristol Laboratories, Syracuse, N. Y. Crystalline monosodium D-α-benzylpenicilloate was prepared as described previously (1, 10 *a*) ϵ-N-(D-α-benzylpenicilloyl)-amino-caproic acid(α-diastereomer) (α-BPO-EACA) was prepared by reaction of NaPG with ϵ-aminocaproic acid and crystallized as its bis-benzylamine salt from ethanol-ether. m.p. = 111–112°C, $[\alpha]_D^{25}$ = + 70.5° (water) (11). The diastereomeric mixture of BPO-EACA (Di-BPO-EACA) was prepared by incubating an aqueous solution



TEXT-FIG. 2. Epimerization of N-(D-α-benzylpenicilloyl)-amines and the reaction of N-(D-α-benzylpenicilloyl)-amines with *p*-chloromercuribenzoate.

of α-BPO-EACA with trace cupric ion, pH 5, 37° under nitrogen for 2½ hours,[2] $[\alpha]_D^{25}$ = + 41.8° (11). Di-BPO-EACA preparations exhibited a new ultraviolet absorption peak at λ

[2] Cupric ion markedly increased the rate of mutarotation of aqueous solutions of BPO-amines. At pH 7.5, 37°, the optical rotation of a 1.0×10^{-2} M solution of α-BPO-EACA containing 1.0×10^{-4} M cupric sulfate fell from α_D = + 0.97° to a stable value of α_D = + 0.50° in 4 hours. Under identical conditions, but in the absence of cupric ion, the optical rotation of a solution of α-BPO-EACA required approximately 100 hours of incubation to reach the stable value of α_D = + 0.50°. In the presence of 1.0×10^{-5} M cupric ion, the mutarotation rate was intermediate between the two rates above. Mutarotation of a 1.0×10^{-2} M solution of D-α-benzylpenicilloic acid at pH 7.5, 37°C, in the absence of cupric ion went to completion within 4 hours. Cupric ion acts presumably by attack on the sulfur atom, increasing the rate of formation of N-(D-α-benzylpenamaldoyl)-amines which appear to be the intermediates through which epimerization takes place (Text-fig. 2.). Epimerization proceeded faster at pH 5 than at pH 7.5. See reference (13) for a discussion of the stereochemistry of benzylpenicilloyl derivatives.

285 mμ which is due to the presence of ε-N-(D-α-benzylpenamaldoyl)-amino-caproic acid formed by rearrangement of BPO-EACA[3] (11) (Text-fig. 2). The above Di-BPO-EACA preparation contained 99.6 per cent BPO-EACA as determined by the penamaldate method (11) and 0.4 per cent penamaldoyl-EACA[3]. Di-BPO-EACA preparations which contained 15 per cent penamaldoyl-EACA were prepared by allowing mutarotation of α-BPO-EACA to proceed under air, in the presence of cupric ion and at pH 7.5. KPG and the BPO-EACA haptens contained no detectible BPE or PSH as determined by methods described previously (1).

P-chloromercuribenzoic acid was a product of Amend Drug and Chemical Co., New York. For use in the penamaldate analytic method, it was dissolved in 0.1 N sodium hydroxide, made up to 1.5×10^{-2} M with 0.05 pH 9.2 carbonate buffer and assayed by the method of Boyer (12). Mercaptoethylamine was furnished by Evans Chemetics, Inc., New York. Other chemicals were of reagent grade. Complete Freunds adjuvant was a product of Difco Laboratories, Detroit. Inoagar No. 2 was obtained from Consolidated Laboratories, Inc., Chicago Heights.

Ultraviolet absorption studies were done with a Zeiss model PMQ 11 spectrophotometer and 1 cm matched quartz cuvettes. Optical rotations were taken with a Schmidt and Haensch polarimeter using a 2 decimeter cell.

The Reaction between BPE and Various Proteins at pH 7.5–8.0.—BPE was reacted with human gamma globulin (HGG), human serum albumin (HSA), gelatin (Gel), and poly-L-lysine (PL) in aqueous solution at pH 7.5–8.0. To obtain maximal conjugation under these conditions, a fivefold molar excess of BPE with respect to lysine ε-amino groups contained in the proteins was generally used.[4] The preparation of a typical conjugate by reaction of HGG and BPE is given:

To 500 mg HGG (0.276 mmoles ε-NH_2) in 50 ml 0.1 M pH 8 phosphate buffer containing 1 mg per ml EDTA (ethylenediaminetetraacetic acid) was added 550 mg of BPE (1.38 mmoles of 85 per cent assay BPE) in 2 ml 95 per cent ethanol. EDTA served to minimize air oxidation of BPE. The mixture was stirred at room temperature for 75 minutes; the reaction went to completion within 45 minutes. pH was maintained between 7.5 and 8.0 by additions of 1 M potassium carbonate. The reaction mixture was centrifuged and the opalescent solution of conjugated protein purified as described below. Protein recovery was 90 per cent.

The Reaction of BPE with Thiolated HGG.—BPE was oxidized together with thiolated HGG in order to prepare a conjugate containing a relatively large number of BPE-SS groups.

[3] The λ 285 mμ peak was destroyed immediately on acidification, and on re-alkalinization, a new absorption peak at λ 265 mμ was formed. These spectral shifts have been described for N-(D-α-benzylpenamaldoyl)-amines (10 *b*). Spectrophotometric assay of N-(D-α-benzylpenamaldoyl)-EACA is based on a molar extinction coefficient at λ_{max} 285 mμ of 23,800 for N-(S-(*p*-mercuribenzoate)-D-α-benzylpenamaldoyl)-ε-aminocaproic acid (Text-fig. 2) (11). It appears to be present mainly in the disulfide form, as its concentration in solutions of BPO-EACA was increased by the presence of oxygen, cupric ion, and higher pH. Also, nitroprusside reactions were negative when carried out on solutions containing 3.0×10^{-3} M penamaldoyl-EACA.

[4] With respect to PL (molecular weight = 180,000; 1400 ε-amino groups per molecule), 625 moles BPE per mole PL was used. The conjugate produced by reaction of PL with BPE was insoluble in the reaction mixture. It was resolubilized by reaction with succinic anhydride as suggested by Eisen (14). This reaction introduces numerous carboxyl groups into the conjugate, thus increasing its solubility at neutral and alkaline pH.

Thiolated HGG was prepared by the method of Singer *et al.* (15) as it was found that the method used by Benesch *et al.* to thiolate gelatin (16) rendered HGG largely insoluble. Ferricyanide was used for the oxidation step, as oxidation of sulfhydryl groups proceeds very rapidly at pH 7 and 4°C (17) thus favoring the formation of BPE-SS groups over aminolysis or hydrolysis of BPE. To a solution of 450 mg of thiolated HGG in 65 ml of cold 0.08 M pH 7 phosphate buffer was added a solution of 246 mg BPE (0.48 mmole) in 3 ml ethanol and a solution of 246 mg (0.75 mmole) potassium ferricyanide in 5 ml. water. The solution was stirred at 4° for 30 minutes; the reaction went to completion within 3 minutes as indicated by the disappearance of the positive nitroprusside test. The conjugate was then purified as described below. Protein recovery was 90 per cent.

The Reaction of D-Penicillamine with Thiolated-HGG.—Thiolated HGG (450 mg) was reacted with 0.48 mmole of D-penicillamine by the identical procedure given above for the reaction of BPE with thiolated HGG and purified by dialysis against amberlite resin (see below). Oxidation of the conjugate with aqueous bromine liberated a ninhydrin-positive compound which exhibited the same R_f on phenol-water paper chromatography as did authentic D-penicillamine sulfonic acid (R_f = 0.18). The formation of penicillamine sulfonic acid indicates that the conjugate contained penicillamine disulfide residues (18). The intensity of the blue spot as compared to the color intensity given by penicillamine sulfonic acid standards indicated that the conjugate contained between 10 and 30 D-penicillamine mixed disulfide residue per mole conjugate.

Purification of the D-Benzylpenicillenic Acid Protein Conjugates.—The side products of the reactions of BPE with native proteins and thiolated HGG were D-benzylpenicilloic acid formed by hydrolysis of BPE (2) and BPE-disulfide which was originally present in the BPE preparation and was also formed by oxidation of BPE (2). Three methods of separating these low molecular weight side products from the conjugates were studied:

(*a*) The conjugates were purified by prolonged dialysis at 4° against large volumes of 0.001 M pH 7 phosphate buffer (7 days with 2 changes of bath fluid per day). This method was found to be unsatisfactory, since after prolonged dialysis, small quantities of BPE-disulfide still remained adsorbed to the conjugates. Table I shows that after prolonged dialysis, PL-BPO still contained 2.6 BPE groups per mole conjugate (1.3 moles BPE disulfide). (*b*) Following 5 days of dialysis against phosphate buffer, the conjugates were dialyzed at 4° against 0.001 pH 7.0 tris-(hydroxymethyl)-aminomethane (tris) buffer in which was suspended an anion exchange resin, amberlite IRA-400. The resin was prepared by cycling through 6 N sodium hydroxide and 3 N hydrochloric acid, washed thoroughly with water and acetone, and equilibrated with 0.001 M pH 7 tris buffer. This method is essentially the same as described by de Weck and Eisen (7). This procedure appeared to be effective in removing BPE-disulfide from the conjugates as the PL-BPO conjugate was completely freed from BPE-disulfide. (Table I). After resin dialysis, the Gel-BPO conjugate still contained 1 group of BPE per 100,000 gm protein (Table I). Gel-BPO may be more difficult to free from absorbed BPE-disulfide because of the tendency of the conjugate to form a gel. (*c*) Direct incubation of the conjugates with amberlite IRA-400 resin at 4° (twice the amount necessary to remove all BPE) was unsatisfactory since it caused a loss of approximately 20 per cent of the conjugate which was firmly absorbed to the resin. The protein molecules removed were presumably the more heavily conjugated since the remaining conjugate contained considerable fewer BPO groups per mole than did the original conjugates (Table I).

The conjugates which were used as antigens in the immunological studies reported below

TABLE I

Analysis of Conjugates of BPE and D-Penicillamine with Various Proteins

Preparation of conjugate*	Abbreviation of conjugate	Purification procedure‡	BPE disulfide§	BPO groups‖
			groups per mole	*per mole*
HGG + BPE	HGG-BPO	a	12.2	37.6
		b	5.1	36.2
		c	2.7	25.0
HGG-BPO, treated with cysteine	HGG-BPO (CSH)	a	0	36.4
		b	0	35.0
		c	0	25.0
HSA + BPE	HSA-BPO	a	5.1	27.4
		b	1.6	27.0
		c	1.0	—¶
Gel + BPE	Gel-BPO	a	3.2	31.6
		b	1.0	29.0
		c	0	—
PL + BPE	PL-BPO	a	2.6	—
		b	0	39.5
		c	0	—
Thiolated HGG + BPE	HGG-SS-BPE	a	24.2	—
		b	17.0	3.6
		c	13.5	—
Thiolated HGG + D-penicillamine	HGG-SS-**D-Penicillamine	b	—	—

* HGG = human gamma globulin, molecular weight = 160,000; HSA = human serum albumin, molecular weight = 69,000; Gel = gelatin, assumed molecular weight = 100,000; PL = Poly-l-lysine, molecular weight = 180,000; Thiolated HGG = HGG enriched with thiol groups by reaction with N-acetylhomocysteine thiolactone, molecular weight = 160,000. See text for preparative details.

‡ a, dialysis against 0.001 M pH 7.2 phosphate buffer for 7 days. b, dialysis as above, followed by further dialysis against amberlite IRA-400 anion exchange resin suspended in 0.001 M pH 7 tris buffer. c, directly incubated with amberlite resin. See text for details. Antigens used for immunologic studies were purified by procedure b.

§ Calculated from corrected absorbancy at λ_{max} 322 mμ and protein concentration by Kjeldahl analysis, corrected for nitrogen contribution of BPE and BPO groups. Molecular weights of conjugates as above. See text for details.

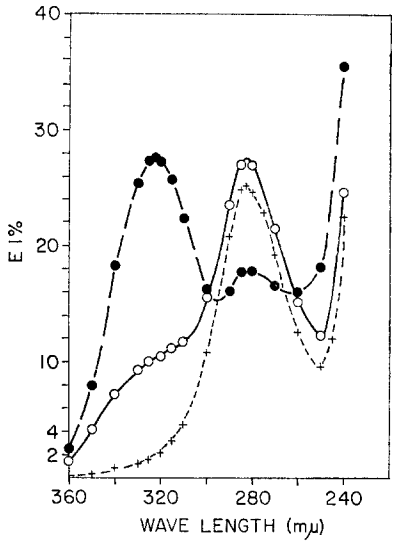
‖ BPO groups assayed by penamaldate method and protein by Kjeldahl analysis corrected as above.

¶ —, analysis not done.

** Contains approximately 10 to 30 groups per mole of D-penicillamine mixed disulfide determined by bromine oxidation and paper chromatography of resulting D-penicillamine sulfonic acid.

were purified by dialysis against amberlite resin. They were finally dialyzed against 0.001 M pH 7.0 phosphate buffer to remove the tris buffer, and the conjugates solutions were stored at −20° or 4°. The conjugates were stable under these conditions; BPE and BPO analyses showed no decrease in the numbers of these groups per mole conjugate after storage at 4° for 4 weeks.

The Detection of BPE-SS-Residues on the Conjugates.—The evidence that the HGG, HSA and thiolated HGG conjugates contained BPE covalently bound through mixed disulfide linkages is: (*a*) These conjugates exhibited an



TEXT-FIG. 3. Ultraviolet absorption spectra of BPE-protein conjugates (listed in Table I). (○), HGG-BPO; (+) HGG-BPO (CSH); (●), HGG-SS-BPE. Protein concentration was determined by Kjeldahl analysis corrected for the nitrogen contribution of BPE and BPO groups. Spectra were expressed as 1 per cent molar extinction coefficient (O.D. per 10 mg protein per ml). Solvent, 0.01 M pH 7.4 phosphate buffer.

ultraviolet absorption peak at λ 322 mu (Text-fig. 3) which persisted despite prolonged dialysis and treatment with an anion exchange resin. This peak has been shown to be due to the BPE grouping (10 *c*). (*b*) The λ 322 mu peak could be destroyed by treatment at pH 7.4 with 0.1 M cysteine, mercaptoethylamine or, to a lesser extent with thioglycollic acid, reagents which are known to cause scission of disulfide linkages (Text-fig. 3) (19). BPE, thus liberated, is rapidly destroyed by hydrolysis (2) or by aminolysis by cysteine (10 *d*). One treatment with cysteine was sufficient to remove all BPE-SS residues from the conjugates. Further treatment of the conjugates with cysteine or mercaptoethylamine, even in the presence of 8 M urea caused no further change in spectrum. Treatment of the conjugates with cysteine did not cause degradation of BPO groups (Table I). BPE-SS groups are covalently bound to native

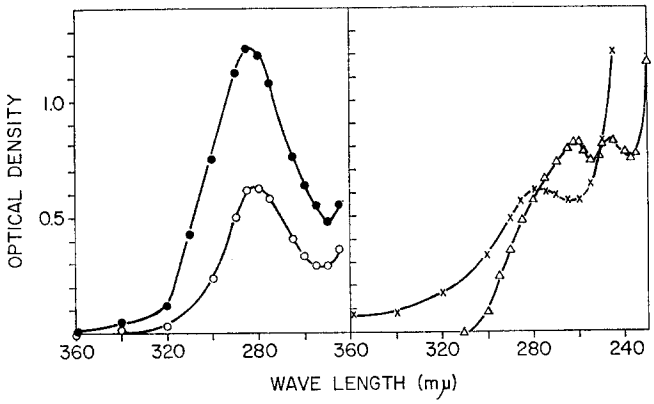
proteins presumably as BPE-SS-Cys groups, and to thiolated HGG mainly as the mixed disulfide of BPE and the N-acetylhomocysteine group introduced into HGG by thiolation (15). The number of BPE-SS residues per molecule of conjugate was calculated from the optical density (O.D.) at λ_{max} 322 mu (assuming ϵ = 26,600 for the BPE-SS residues, reference 9) and the protein concentration as determined by micro-Kjeldahl analysis (8 *a*). The protein concentration was corrected for the nitrogen contribution of BPE and BPO groups bound to protein; this correction was approximately 3 to 5 per cent. The O.D. at λ_{max} 322 mu was corrected for the O.D. at this wave length exhibited by the conjugate after the BPE groups had been removed by reduction with cysteine. For conjugates containing large numbers of Di-BPO groups, this correction amounted to 7 to 14 per cent of the O.D. at λ_{max} 285 mu (Text-fig. 3).

The Detection of Di-BPO-Lysine Residues on the Conjugates.—The evidence that the conjugates contained covalently bound BPO residues is: (*a*) Alkaline hydrolysis of the protein conjugates (purified by dialysis and amberlite treatment) liberated D-benzylpenicilloic acid, the expected hydrolytic product of Di-BPO-Lys groups.

A mixture of 2.0 ml of HGG-BPO (CSH) (3.5 mg per ml) and 0.2 ml of 2 N sodium hydroxide was heated in a boiling water bath for 15 minutes. Aliquots of 0.10 ml of the reaction solution were analyzed by descending paper chromatography along with non-hydrolyzed conjugate and authentic D-benzylpenicilloic acid which served as standards. The chromatogram was developed with a solvent mixture of ethanol, *n*-butanol and ammonium carbonate buffer prepared as described by Shepartz and Johnson (20). The dried chromatogram was sprayed with the mercuric chloride-arsenomolybdate reagent described by Pan (21) which is reduced at room temperature by penicilloic acids to form the intense blue color of reduced Nelson's reagent. The chromatogram of the hydrolyzed conjugate exhibited a faint blue spot with the same R_f as that of D-benzylpenicilloic acid (R_f = 0.35). This spot was not found in the chromatogram of the unhydrolyzed conjugate. By this method alkaline hydrolysis of crystalline α-BPO-EACA[1] was found also to yield D-benzylpenicilloic acid.

(*b*) Treatment of an aqueous solution of the conjugates at pH 9.2 with mercuric chloride resulted in an immediate marked increase in ultraviolet absorbancy at λ_{max} 285 mu. Acidification of this solution immediately abolished this extra absorbancy, and realkalinization caused the instantaneous formation of a new strong ultraviolet absorption peak at λ 262 mu (Text-fig. 4). These spectral shifts have been described previously for BPO-amines (10 *b*) and are due to the rearrangement of BPO-amines to penamaldoyl-amines[1] (Text-fig. 2) which are degraded by acid to benzylpenaldoyl-amines (10 *b*). The extra absorption peak at λ 245 mu in the realkalinized solution of the conjugate (Text-fig. 4) may be due to the D-penicillamine mercaptide ion. D-penicillamine is formed by the acid hydrolysis of penamaldoyl-amine groups and in aqueous alkaline solution exhibited an absorption peak at λ 240 mu. Crystalline α-BPO-EACA was found to exhibit the identical spectral shifts when treated as described above. The spectral shifts are shown in Text-fig. 4.

(*c*) The HGG and HSA-BPO conjugates were assayed for ε-amino groups by formol titration (22) and for BPO groups by the penamaldate method described below. Table II shows that the number of BPO groups introduced into the proteins was in excellent agreement with the number of ε-amino groups bound. These data indicate that BPO groups are bound predominantly as BPO-Lys groups.[5] The reaction of BPE with lysine ε-amino groups would yield a diasteromeric mixture of BPO-Lys groups (Di-BPO-Lys), since two new asymmetric carbon centers (marked (*) in Text-fig. 1) are formed during the reaction. The reaction of BPE with lysine ε-amino groups to yield Di-BPO-



TEXT-FIG. 4. The spectral shifts exhibited by HGG-BPO (CSH), (○), represents HGG-BPO (CSH) at 0.25 mg/ml in 0.01 M pH 9.2 carbonate buffer; (●), represents the preceding solution treated with one molar equivalent (BPO groups) of mercuric chloride; (×), represents the preceding solution acidified with 2 volumes of concentrated hydrochloric acid; (△) represents the proceeding solution, the acid removed by dialysis, and the pH adjusted to > 12 with sodium hydroxide. The optical densities were corrected for dilutions incurred by addition of reagents.

Lys groups is analogous to the previously described reaction of BPE with β-alanine to form the diastereomeric mixture of N-(D-α-benzylpenicilloyl)-β-alanine (2).

Quantitative Assay of BPO Groups Bound in the Conjugates (Penamaldate Method).—Quantitative assay of BPO groups in the conjugates is based on the penamaldate reaction which is described in detail in another report (11). Briefly, it was found that in aqueous solution at pH 8.5 to 10, treatment of the crystalline low molecular weight BPO-amines with 4 to 50 molar

[5] Free amino groups of terminal amino acids may also react with BPE. The possibility that BPE may react also with tyrosine hydroxyl groups, histidine groups, and other groups on protein has not been evaluated here. From Table II, it appears that under the conditions employed here, the BPO groups were bound predominantly as Di-BPO-Lys. However, from the data given below under Ultraviolet Absorption Spectra of Conjugates, it appears that some BPO groups are bound as ε-N-(N^4-(D-α-benzylpenicilloyl)-D-α-benzylpenamaldoyl)-lysine groups (Text-fig. 6).

equivalents of *p*-chloromercuribenzoate (PCMB)[6] caused the immediate formation of a new ultraviolet absorption peak at λ 285 mu with $\epsilon = 23{,}800 \pm 2$ per cent (11). This peak was demonstrated to be due to the formation of N((S-*p*-mercuribenzoate)-D-α-benzylpenamaldoyl)-amines (Text-fig. 2) (11). Under these conditions, BPO-amine concentration versus O.D. at λ_{max} 285 mu obeyed Beer's Law. This analytical method appears to be valid also for the quantitative determination of BPO groups on BPO-conjugates as indicated by the following observations: (*a*) Treatment of the BPO-conjugates with PCMB caused the formation of λ 285 mu peak (Text-Fig. 4). (*b*) Treatment of various native proteins did not cause an increased absorbancy at λ 285 mu, nor did the native proteins interfere with the development of the λ 285 mu peak by reaction of PCMB with BPO-amines. (*c*) The number of BPO groups per mole conjugate as determined by the penamaldate method was in good agreement with the number of ε-amino groups of the protein that were bound (Table II).

TABLE II

Comparison between the Number of BPO Groups Introduced into Proteins by Reaction with BPE and the Number of ε-Amino Groups Bound

Proteins analyzed*	ε-Amino groups‡	ε-Amino groups bound	BPO groups§
	number/mole protein	*number/mole protein*	*number/mole protein*
HGG	88	0	0
HGG-BPO	51	37	37
HSA	59	0	0
HSA-BPO	34	25	27

* See legend of Table I for abbreviations.

‡ ε-Amino groups by formol titration (22) and protein analysis by Kjeldahl corrected for nitrogen contribution of BPE and BPO groups. Molecular weights of proteins: HGG and HGG-BPO, 160,000; HSA and HSA-BPO, 69,000.

§ BPO analysis by penamaldate method and protein by Kjeldahl corrected as above.

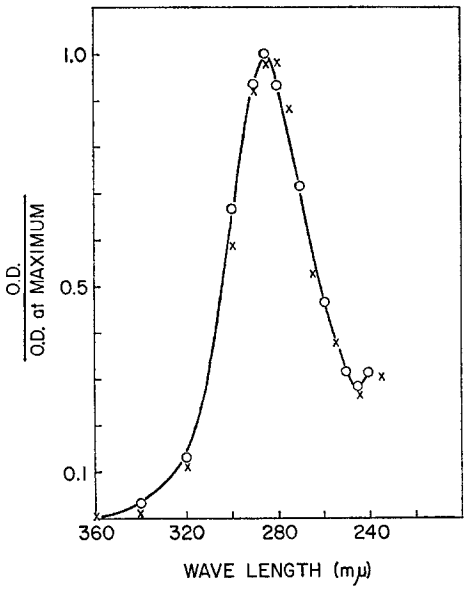
In practice, 5.00 ml of a solution of the conjugate in 0.05 M pH 9.2 carbonate buffer which was approximately 3.0×10^{-5} M with respect to BPO groups was treated with 0.15 ml of 1.50×10^{-2} PCMB solution in the carbonate buffer. The O.D. at λ_{max} 285 mu was measured between 5 and 15 minutes[7] after the reactants were mixed. From this O.D. was subtracted the O.D. at λ 285 mu of the conjugate prior to PCMB treatment plus the O.D. of 4.5×10^{4} PCMB (0.100 O.D. units). The remainder was corrected for dilution (multiply by 1.03), and BPO concentration was calculated from this corrected O.D. value assuming the molar extinc-

[6] Treatment of BPO-amines with mercuric chloride also caused the immediate formation of the λ 285 mμ peak. However, especially in the presence of excess reagent, the absorption peak formed in the presence of mercuric ion was considerably less stable than was the peak formed in the presence of PCMB. Accordingly, PCMB appears to be more useful than is mercuric chloride for the penamaldate analysis.

[7] The intense absorption maximum at λ 285 mμ formed immediately on mixing the reactants, after which the absorbancy at λ_{max} 285 mμ fell slowly. Between 5 and 15 minutes after the reactants were mixed, the O.D. at λ_{max} 285 mμ decreased approximately 3 per cent. The value $\epsilon = 23{,}800$, was calculated from the average of the O.D. at 5 and 15 minutes after the reactants are mixed, the maximum error incurred from the slight instability of the absorption peak was 1.5 per cent.

tion coefficient of protein-bound BPO groups to be 23,800 (11). The number of BPO groups per mole conjugate was calculated from this value and the protein concentration as determined by Kjeldahl analysis (8 *a*) corrected for the nitrogen contribution of BPO and BPE groups.

The Numbers of BPE-SS Groups and Di-BPO Groups in the Conjugates.—The numbers of Di-BPO groups and BPE-SS groups introduced into the various

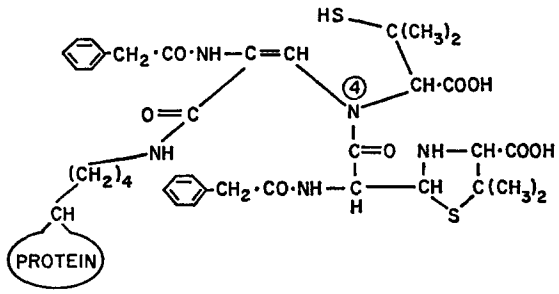


TEXT-FIG. 5. Ultraviolet absorption spectrum of N-(D-α-benzylpenamaldoyl)-ε-aminocaproic acid (O) and the substituted amino acids responsible for the extra absorbancy at λ_{max} 285 mμ exhibited by HGG-BPO (CSH) (×). The former was obtained by treatment of N-(D-α-benzylpenicilloyl)-ε-aminocaproic acid with 1 molar equivalent of mercuric chloride in 0.01 M pH 9.2 carbonate buffer. The latter was obtained by correcting the spectrum of HGG-BPO (CSH) for the contributions of protein and BPO groups. Optical densities are expressed as a fraction of the O.D. at λ_{max} 285 mμ.

conjugates are shown in Table I. The numbers of BPE-SS groups introduced into the native serum proteins are in the range of the numbers of sulfhydryl and disulfide linkages per mole of serum proteins available for reaction under "mild" conditions; *i.e.*, as—SH, 3 for HSA (5). The numbers of Di-BPO-Lys groups introduced into HGG-BPO (36 groups per mole) and HSA-BPO (27 groups per mole) are in the range introduced under "mild" conditions (23). Greater numbers of these groups, up to 55 per mole, were introduced into HGG by reaction with BPE at pH 9.0–9.5, 10 per cent ethanol, and with vigorous stirring; *i.e.*, conditions which favor denaturation. The almost complete

derivatization of ϵ-amino groups achieved with gelatin would be expected for this mixture of denatured protein fragments.

Electrophoresis and Ultracentrifugation of HGG-BPO (CSH) Conjugate.—The HGG-BPO (CSH) conjugate exhibited more rapid migration toward the anode on paper electrophoresis at pH 8.6 than did native HGG; it migrated with the mobility of an α-globulin. This is expected for a protein conjugate whose ϵ-amino groups are blocked by negatively charged BPO groups, thus lowering its isoelectric point. Ultracentrifugation of the conjugate showed it to be composed almost entirely of 7S sedimenting material but containing a trace of material sedimenting at 8S to 10S. This pattern was identical with that exhibited by the native HGG preparation.



TEXT-FIG. 6. The ϵ-N-(N⁴-(D-α-benzylpenicilloyl)-D-α-benzylpenamaldoyl)-lysine residue in the BPO-conjugates.

Ultraviolet Absorption Spectra of the Conjugates.—The ultraviolet absorption spectra of some typical conjugates are shown in Text-fig. 3. The HGG-BPO conjugates, as well as the HSA-BPO, Gel-BPO-, and PL-BPO, exhibited a considerably greater absorbancy at λ_{max} 285 mu than did the native proteins (Text-fig. 3). This extra absorbancy is not due to Di-BPO-Lys groups as BPO-amines have essentially no absorption at λ 285 mu (10 *e*). That PL-BPO also exhibited this extra λ_{max} 285 mu absorbancy indicated that the λ 285 mu peak was due to a substituted lysine ϵ-amino group. The ultraviolet absorption spectrum of the HGG-BPO (CSH) conjugate was corrected for the ultraviolet absorption of HGG and BPO groups to yield the absorption spectrum of the substituted amino acids responsible for this extra absorbancy at λ_{max} 285 mu. Text-fig. 5 shows that this spectrum is not significantly different from that of N-(D-α-benzylpenamaldoyl)-EACA (see Text-fig. 2). The spectral data and the observation that the λ_{max} 285 mu absorption was stable to acidification suggest that the extra absorbancy may be due to ϵ-N-(D-α-benzylpenamaldoyl)-lysine groups whose N④ nitrogen atoms have been acylated with 1 mole of BPE (Text-fig. 6). The presence of this group would be consistent with the

agreement found between the number of BPO groups introduced into the conjugates and the number of ε-amino groups bound (Table II). The HGG-BPO (CSH) conjugate (Text-fig. 3) requires 7.3 of these groups per mole of conjugate to account for the extra absorbancy at λ 285 mu.

TABLE III

*Immunization of Rabbits with KPG and with a KPG-NRS Incubate**

Immunizing antigen*	Animal No.	Ring precipitation tests with‡				Skin tests with§		
		HGG-BPO (CSH)	HGG-SS-BPE	HGG-S-S-D penicillamine	HGG	HGG-BPO (CSH)	HGG-BPO	HGG
KPG	1	1+	1+	0	0	1+	1+	0
	2	1+	1+	0	0	1+	1+	0
	3	1+	1+	0	0	2+	2+	0
	4	2+	2+	0	0	2+	2+	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0
KPG-NRS	7	2+	—	—	0	—	—	—
	8	2+	—	—	0	—	—	—
	9	3+	—	—	0	—	—	—
	10	2+	—	—	0	—	—	—
	11	3+	—	—	0	—	—	—
	12	3+	—	—	0	—	—	—

* KPG, potassium penicillin G; KPG-NRS—KPG incubated with normal rabbit serum at 37°C, pH 7, 3 hours. See text for method of immunization.

‡ The pooled antiserum for each animal was overlayed in a capillary tube with a 0.50 mg/ml solution of the conjugate in buffered saline. Readings at 15 minutes were: 0, no reaction; 1+, slight precipitation at the interphase; 2+ moderate precipitation; 3+, marked precipitation and flocculation. For abbreviations, see legend, Table I. (—) test not done.

§ Injection into the skin of the flank of 200 μg protein in 0.10 ml of buffered saline. Reactions at 4 hours were graded: 0, edema up to 5 mm, no hemorrhage; 1+, edema of 10–20 mm, slight hemorrhage; 2+, edema 20–30 mm, moderate hemorrhage. Three non-sensitized rabbits gave 0 reactions to these proteins.

Immunization of Rabbits with Benzylpenicillin (PG).—Six albino rabbits weighing 2500 gm were immunized by injection of potassium penicillin G (KPG) emulsified in complete Freund's adjuvant. A similar method has been reported by Josephson (6). On the 1st and 10th day the animals received a total dose of 100 mg of KPG emulsified in 1 ml water and 1 ml adjuvant injected into all four foot-pads and intradermally in multiple sites in the dorsal skin. On the 18th, 20th, 22nd, 24th, and 26th day, the rabbits received 100 mg KPG in 2.0 ml of water intravenously and 300,000 units of aqueous procaine penicillin-G (a product of Pfizer Laboratories, Brooklyn) injected into the gluteal muscle. On the 32nd, 34th, and 36th day, the animals were bled from the ear artery by the method given by Leskowitz and Waksman (24). The three bleedings from each animal were pooled and ring precipitation tests were done on each serum pool. Four days after the last bleeding, skin tests were performed by intradermal injection of HGG-BPO (CSH), HGG-S-S-BPE, and HGG.

The results of the ring tests and the skin tests are given in Table III. Four of the six rabbits developed serum antibodies and Arthus skin reactivity specific for the conjugates. In another experiment six rabbits were immunized by this schedule and with the dosage of KPG and adjuvant described above, but with a solution of KPG in normal rabbit serum and 0.15 M NaCl (1:1 by

TABLE IV

*Qualitative Precipitation Tests on Pooled Rabbit Anti-Penicillin G Sera and Hapten Inhibition of Precipitation**

Testing antigen*	Sera tested‡		
	Pooled normal rabbit sera	Pooled rabbit anti-PG sera	Pooled rabbit anti-(PG-NRS) sera
HGG-BPO (CSH)	0	2+	3+
HGG-BPO (CSH) + Di-BPO-EACA (hapten)	0	0	0
HGG-BPO	0	2+	—§
HSA-BPO	0	1+	2+
Gel-BPO	0	1+	—
PL-BPO	0	2+	3+
HGG-S-S-BPE	0	1+ to 2+	2+
HGG-S-S-BPE + Di-BPO-EACA (hapten)	0	0	0
HGG-S-S-penicillamine	0	0	0
HGG	0	0	0

* See Table I for abbreviations and analysis of the antigens. Di-BPO-EACA was the 0.4 per cent penamaldoyl preparation (see Materials). Final concentrations of hapten in the inhibited mixtures was 1.0×10^{-3} M. Di-BPO-EACA did not inhibit precipitation of rabbit antibovine γ-globulin by bovine γ-globulin.

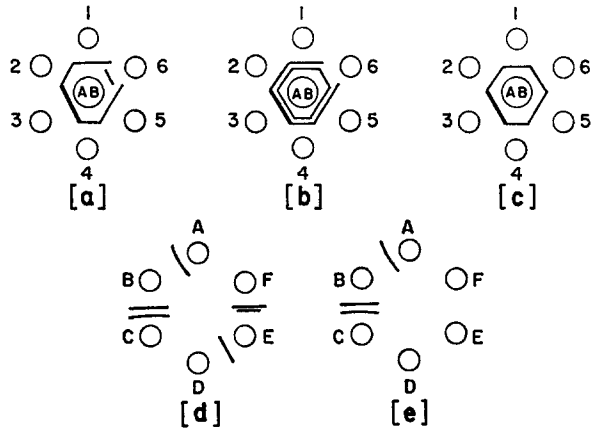
‡ 0.5 ml. serum mixed with 175 μg antigen (total volume = 1.0 ml) and the mixture stored at 4° for 24 hours and centrifuged. Precipitates were graded: 0, no precipitate; 1+, mild; 2+ moderate; 3+, marked precipitation.

§ Test not done.

volume) which had been incubated at pH 7 and 37°C for 3 hours. Under these conditions the sera of all six rabbits so immunized exhibited strongly positive ring precipitation tests which the HGG-BPO (CSH) antigen and negative reactions to HGG (Table III).

Precipitation Analysis of Rabbit Anti-penicillin Sera.—The sera from rabbits 1 to 4 (Table III) were pooled (anti-PG pool) as were the sera of rabbits 7 to 12 (anti-(PG-NRS) pool). Qualitative precipitation tests and hapten inhibition of precipitation were carried out on the two pools of antisera using the conjugates listed in Table I as antigens. Table IV shows that all the conjugates

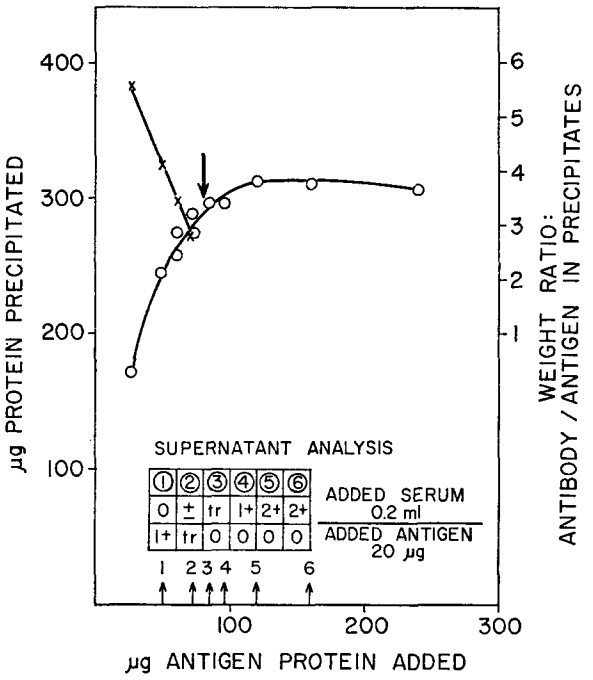
containing Di-BPO groups were capable of specifically precipitating both the anti-PG and anti-(PG-NRS) pooled antisera. For both anti-PG and anti-(PG-NRS) sera, Di-BPO-EACA hapten at 1.0×10^{-3} M concentration completely inhibited precipitation of the antisera by the HGG-BPO (CSH) and HGG-S-S-BPE antigens. This inhibition was specific as higher concentrations of the hapten (1×10^{-2} M) did not inhibit precipitation of rabbit antibovine γ-globulin by bovine γ-globulin.



TEXT-FIG. 7. Agar-gel diffusion analysis of the pooled rabbit anti-PG serum. Each figure represents an agar plate incubated at room temperature for 48 hours (Fig. *a*, *c*) or for 96 hours (Fig. *b*, *d*, *e*). Fig. *e* represents an agar plate containing Di-BPO-EACA, at 2.0×10^{-3} M concentration. In Figs. *a*, *b*, *c*, the wells contained: *AB*. Rabbit anti-PG serum concentrated threefold; *1*. HGG-BPO (CSH); *2*. HGG-BPO; *3*. PL-BPO; *4*. HGG-BPO (CSH); *5*. HSA-BPO all at 450 μg per ml. in B.S.; *6*. HGG-SS-BPE in Figs. *a*, *b* at 700 μg per ml and in Fig. *c* at 2.20 mg per ml. In Figs. *d*, *e* the wells contained: *A*. Rabbit anti-dinitrophenylbovine serum albumin; *B*. Dinitrophenyl-bovine γ-globulin, (7.4 DNP residues per mole), 22.0 mg per ml; *C*. Rabbit Anti-bovine γ-globulin; *D*. HGG-SS-BPE, 2.50 mg per ml.; *E*. Rabbit anti-PG serum (concentrated three-fold); *F*. HGG-BPO (CSH), 350 μg per ml.

Agar-Gel Diffusion Studies and Hapten Inhibition of Precipitation in Gel.— Agar-gel double diffusion analyses of the rabbit pooled anti-PG and the rabbit pooled anti-(PG-NRS) sera with the various conjugates as antigens were carried out by the Petri dish method of Ouchterlony (25) using plates made with 0.5 per cent ionagar dissolved in 0.15 M sodium chloride. The inhibited plate contained Di-BPO-EACA hapten dissolved in the liquid gel at a concentration of 2.0×10^{-3} M. The anti-PG serum was concentrated by lyophylization and reconstitution with water to one-third the original volume. The anti-(PG-NRS) serum was used as such. The results of gel-diffusion analysis of the anti-PG serum are shown in Text-fig. 7 and Fig. 1. The pattern which is formed by the HGG-SS-BPE and the HGG-BPO (CSH) lines at 48 hours (Text-fig. 7 *a*)

may be attributed to the considerably fewer Di-BPO groups present in HGG-SS-BPE (3.6 per mole) than present in HGG-BPO (CSH) (36 per mole). When the concentration of HGG-SS-BPE was increased to five times that of HGG-BPO (CSH) a pattern of complete identity was obtained (Text-fig. 7 *c*). The double precipitation lines seen in Text-fig. 7 *b* have been observed also by

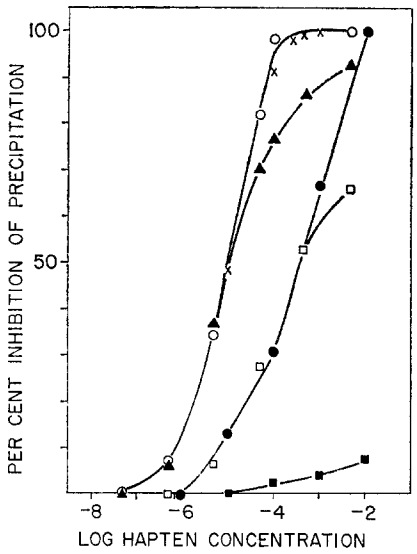


TEXT-FIG. 8. Specific precipitation of pooled rabbit anti-PG serum. Each tube contained 0.50 ml of pooled antiserum from rabbits immunized with KPG (see text). The test antigen was HGG-BPO (CSH) (Table I). Total volume per tube = 2.0 ml. (○) represents μg protein precipitated (antigen + antibody); (×) represents weight ratios: antibody/antigen in the precipitates. Antibody (μg) was calculated by subtracting the μg antigen added from the μg total precipitate (in the zone of antibody excess). The arrow (↓) indicates the equivalence point.

Josephson and Franklin (26) who suggested that the line nearer the antibody well is due to a 19S antibody, and the other line to a 7S antibody. The formation of both these lines was specifically inhibited by Di-BPO-EACA (Text-fig. 7 *d*, *e*), indicating that both lines are specific for the BPO group.

Quantitative Precipitin Analysis of Rabbit anti-PG Sera.—Quantitative precipitation analysis of the pooled anti-PG sera with HGG-BPO (CSH) as antigen revealed an antibody content of 420 μg per ml of antibody protein.

This figure is in the range of antibody contents achieved by Gell on immunization of rabbits with several low molecular weight chemicals (27). The precipitin curve is shown in Text-fig. 8 and the procedural details are given below. The precipitation curve, supernatant analysis, and antibody/antigen ratios in the



TEXT-FIG. 9. Specific inhibition of precipitation by haptens. Precipitation reactions were set up at the equivalence point. Each tube contained 0.50 ml. pooled rabbit anti-PG serum, 80 μg. HGG-BPO (CSH) antigen, and hapten in various quantities. Total volume = 2.0 ml; diluent was buffered saline (pH 7.2). The uninhibited control yielded 290 μg protein precipitate.

$$\text{per cent inhibition} = \frac{100\ (\mu\text{g precipitate of uninhibited control} - \mu\text{g precipitate of test mixture})}{\mu\text{g precipitate uninhibited control}}$$

(○) represents Di-BPO-EACA (15 per cent penamaldoyl preparation); (×) represents Di-BPO-EACA (0.4 per cent penamaldoyl); (▲) α-BPO-EACA; (●) D-α-benzylpenicilloic acid; (□) KPG; (■) benzylamine. None of the haptens inhibited precipitation of rabbit anti-bovine γ-globulin by bovine γ-globulin.

precipitates show that this system behaves as do classical precipitating single antigen-antibody systems (8 *b*).

To 0.50 ml of antiserum was added variable quantities of HGG-BPO (CSH) antigen in B.S.[1] and the volumes adjusted to 2.00 ml with B.S. Reaction mixtures as well as antiserum and antigen blanks were set up in duplicate. The tubes were incubated at 37° for 30 minutes and at 4° for 36 hours. Precipitates were washed 3 times with ice cold saline, air-dried, and dissolved in 0.1 N sodium hydroxide. Aliquots were then analyzed for protein by the Folin method (28). The Folin method could be used as standardization curves with HGG-BPO (CSH) and rabbit γ-globulin (FxII) (Pentex, Inc., Kankakee, Illinois) showed that for each protein the color values at λ_{max} 750 mu per μg protein differed by less than 3 per cent.

Quantitative Hapten Inhibition of Precipitation.—Quantitative inhibition experiments were carried out on the pooled rabbit anti-PG sera. The haptens dissolved in B.S. were mixed with 0.50 ml serum and the antigen (HGG-BPO) (CSH) was added in amount required to reach the equivalence point in the uninhibited control (80 μg antigen protein). The total volume was 2.00 ml made up with B.S. Inhibition mixtures, uninhibited controls and antiserum and antigen blanks were set up in duplicate. After incubation at 37°C for 30 minutes and 36 hours at 4°C the precipitates were washed and analyzed as described above.

The inhibition curves given by various haptens are shown in Text-fig. 9. Di-BPO-EACA exhibited strong inhibition of precipitation; 50 per cent inhibi-

TABLE V

*Testing of Pooled Rabbit Anti-PG Serum with HGG-BPO (CSH) Antigen by PCA in Guinea Pigs and Specific Inhibition of PCA by Haptens**

Pooled rabbit anti-PG serum dilution	Average diameter of PCA Reaction, *mm*															
	Uninhibited animals					Inhibition with 10 mg α-BPO-EACA‡					Inhibition with 10 mg Di-BPO-EACA‡					
1/200	28	25	16	14	13	12	13	12	13	12	0	0	0	0	0	0
1/500	18	15	10	tr	0	tr	0	0	0	0	0	0	0	0	0	0
1/1000	16	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/2000	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit anti-ovalbumin 0.04 μg. antibody N	25	18	8	tr	tr	20	18	20	25	18	25	20	15	20	25	20

* Latent period between sera and antigen injection, 6 hours.

Antigen mixture containing 200 μg HGG-BPO (CSH), 75 μg ovalbumin and 0.5 ml Evans blue total volume = 1 ml) was injected intravenously. For the inhibition studies, hapten in 1.0 ml saline was injected intravenously 5 minutes prior to injection of antigenic mixture. Animals were killed 12 minutes after injection of antigen. Each vertical line represents an individual 250 to 300 gm guinea pig.

‡ α-BPO-EACA and Di-BPO-EACA = α-diastereomer and diastereomeric mixture of N-(D-α-benzylpenicilloyl)-ε-aminocaproic acid. Di-BPO-EACA was 0.4 per cent penamaldoyl preparation.

tion at 1.0×10^{-5} M concentration and 100 per cent inhibition at 4.0×10^{-4} M concentration. At the higher concentrations of the haptens, the diastereomeric mixture of BPO-EACA was a more effective inhibitor than was the α-diastereomer of BPO-EACA. The equal effectiveness of the two forms of BPO-EACA at the lower concentrations may be ascribed to the ability of the α-diastereomer to undergo epimerization in the presence of cupric ion contained in the serum to yield the diastereomeric mixture of BPO-EACA.[2] Since the effect of cupric ion increases as the ratio: concentration of cupric ion/concentration BPO-EACA increases[2] the lower concentrations of α-BPO-EACA would undergo epimerization to a greater extent than would the higher concentrations. Although D-α-benzylpenicilloic acid was used as an inhibitor, in

TABLE VI

*Testing of Pooled Rabbit Anti-PG Serum with HGG-S-S-BPE Antigen by PCA in Guinea Pigs and Specific Inhibition of PCA by Haptens**

Anti-PG serum dilution	Average diameter of PCA reaction, *mm*	
	Uninhibited animals	Inhibition with 10 mg Di-BPO-EACA
1/50	15 8 15 16 16	0 0 0 0 0
1/100	12 tr 10 13 15	0 0 0 0 0
1/200	10 tr 10 13 15	0 0 0 0 0
1/500	7 0 tr tr 7	0 0 0 0 0
1/1000	7 0 tr tr 7	0 0 0 0 0
1/2000	0 0 0 0 0	0 0 0 0 0

* Antigen mixture: 250 μg HGG-SS-BPE + 0.5 ml 1 per cent Evans blue; total volume: 1 ml in saline. Latent period, 5 hours. See legend, Table V for experimental procedures. Each vertical line represents an individual animal.

TABLE VII

*Testing of Pooled Rabbit Anti-PG Sera and Pooled Rabbit Anti-(PG-NRS) Sera with HGG-S-S-D-Penicillamine Antigen by PCA in Guinea Pigs**

Anti serum‡ dilution		Average diameter of PCA reaction, *mm*	
		HGG-S-S-D-penicillamine 500 μg§	HGG-BPO (CSH) 200 μg§
Anti-PG serum	1/10	0 0 0 0	25 30
	1/50	0 0 0 0	15 20
	1/100	0 0 0 0	15 20
Anti-(PG-NRS) serum	1/50	0 0 0 0	15 20
	1/100	0 0 0 0	15 20

* HGG-S-S-D-penicillamine antigen contains 10 to 30 groups of D-penicillamine mixed disulfide per mole. Latent period, 5 hours. See legend Table V for experimental procedures. Each vertical line represents an individual animal.

‡ For description of antisera, see text.

§ Thirty minutes after injection of the HGG-SS-D-penicillamine antigen, two negatively reacting animals were re-injected with HGG-BPO-(CSH) antigen (Table I). Positive reactions appeared within 4 minutes.

serum solution it probably exists mainly as the diastereomeric mixture since the α-diastereomer rapidly undergoes epimerization, approximately 25 times faster than does α-BPO-EACA.[2]

The inhibition curves obtained with the two Di-BPO-EACA preparations containing concentrations of penamaldoyl-EACA of 0.4 per cent and 15 per cent of the original BPO-EACA concentration did not appear to differ signifi-

cantly. D-Benzylpenicilloic acid was 1/40th as effective an inhibitor (mole for mole) as was BPO-EACA; 50 per cent inhibition for D-benzylpenicilloic acid was achieved at 4.0×10^{-4} M concentration and 100 per cent inhibition at 1.0×10^{-2} M. PG exhibited incomplete inhibition of precipitation. Benzylamine which is present in the preparations of BPO-EACA caused less than 5 per cent inhibition at 1.0×10^{-3} M concentration. None of the haptens, in the concentrations used, inhibited the precipitation of rabbit anti-bovine-γ-globulin by bovine γ-globulin.

TABLE VIII

*Detection of Antigenic BPO-Conjugate in the Serum of a Patient (V.C.) Treated with KPG by PCA Reaction in Guinea Pigs**

Anti-PG serum dilution		Average diameter of PCA reaction, *mm*	
		0.5 ml V.C. serum (employed as antigen)	10 mg Di-BPO-EACA‡ followed by 0.5 ml V.C. serum (antigen)
Human* (V.O.)	1/10	11 18 0	10 (pale) 0 0 0
	1/40	0 0 0	0 0 0 0
	1/60	0 0 0	0 0 0 0
Pooled rabbit anti-PG serum	1/50	15 20 18	10 (pale) 0 0 0
	1/100	10 15 15	0 0 0 0
	1/500	0 0 8	0 0 0 0

* V.C. serum from a patient being treated with 25 gm (40 million units) of KPG per day. Serum was dialyzed immediately after being obtained from patient. V.O. serum from a patient with recent allergic reaction to PG (see Table IX). Latent period between intradermal serum injections and antigen (0.5 ml V.C. serum + 0.5 ml 1 per cent Evans blue), 5 hours. Di-BPO-EACA in 1.0 ml normal saline injected intravenously 5 minutes prior to injection of antigen. Animals were killed 12 minutes after injection with antigen. Positive reactions appeared in 4 minutes. Each vertical line represents an individual animal.

‡ 0.4 per cent penamaldoyl preparation.

Analysis of Rabbit Anti-Penicillin Sera by Passive Cutaneous Anaphylaxis (PCA) in the Guinea Pig and Inhibition of PCA with Haptens.—PCA analysis and hapten inhibition of PCA was carried out on the pooled rabbit anti-PG and anti(PG-NRS) sera by the methods described previously (29–31). The results are shown in Tables V to VII. Prior titration with HGG-BPO (CSH) antigen showed that maximal reactions were obtained with 200 to 300 μg of antigen. Using 200 μg HGG-BPO (CSH) as antigen, the pooled rabbit anti-PG sera gave positive PCA reactions to a dilution of 1:2000 to 1:2500. Prior injection of 10 mg of α-BPO-EACA (Text-fig. 2) caused strong but incomplete inhibition of the PCA reaction, whereas prior injection of 10 mg of Di-BPO-EACA (0.4 per cent penamaldoyl preparation) caused complete inhibition of

PCA (Table V) (Fig. 2). With 250 μg HGG-SS-BPE as antigen, the pooled rabbit anti-PG sera showed positive responses to a dilution of 1:1000. Prior injection of 10 mg of Di-BPO-EACA resulted in complete inhibition of PCA reaction (Table VI). The inhibition of PCA reaction by Di-BPO-EACA was specific as it did not inhibit PCA given by the ovalbumin-rabbit-antiovalbumin system (Table V). Antibodies directed against D-penicillamine mixed disulfide groups could not be detected by PCA reaction in both the anti-PG and anti-(PG-NRS) pools (Table VII). The pooled rabbit anti-(PG-NRS) sera gave positive reactions to a dilution of 1:3500 with 200 μg HGG-BPO (CSH) as antigen. With the pooled anti-(PG-NRS) as well as the anti-PG sera, PCA reactions provoked by the HGG-BPO(CSH) and HGG-SS-BPE antigens were completely inhibited by prior injection of 10 mg of Di-BPO-EACA.

TABLE IX

*Detection of Anti-BPO Antibody in Serum of a Patient with a Recent Urticarial Reaction to PG by PCA Reaction in Guinea Pigs**

V.O. serum dilutions	Average diameter of PCA reactions, *mm*	
	HGG-BPO (CSH) 500 μg	10 mg Di-BPO-EACA‡ followed by 500 μg. HGG-BPO(CSH)
1/20	20 20 tr 0	0 0 0 0
1/40	12 15 0 0	0 0 0 0
1/60	0 12 0 0	0 0 0 0

* Latent period, 5 hours. Each vertical line represents an individual animal. See legend, Table VIII for hapten inhibition technique.

‡ 0.4 per cent penamaldoyl preparation.

Studies on Human Beings; Detection of Antigenic BPO-Conjugates in the Serum of a Patient Treated with Large Doses of Penicillin G.—Serum from a patient (V.C.) under treatment for subacute bacterial endocarditis with 40 million units (25 gm) KPG intravenously per day was immediately dialyzed thoroughly against saline and assayed for antigenic activity by PCA with hapten inhibition of PCA (29–31). The data in Table VIII showing the effectiveness of V.C. serum as an antigen and the inhibition of PCA by Di-BPO-EACA hapten demonstrate the presence of non-dialyzable antigenic BPO-conjugates in this serum.

Detection of Anti-BPO Antibodies in the Serum of a Patient with a Recent Allergic Reaction to PG.—Serum obtained from a patient (V.O.) 6 days after an urticarial reaction to PG was assayed for anti-BPO antibodies by PCA. The data in Table IX showing complete inhibition of PCA reactions by prior injection of Di-BPO-EACA demonstrate the presence of anti-BPO antibodies

in this serum. Sera from five other patients with histories of recent urticarial reactions while on PG gave negative results by this method when tested with the HGG-BPO (CSH) and HGG-SS-BPE antigens.

Skin Testing of Patients with Histories of Allergic Reactions to PG, and Specific Hapten Inhibition of the Wheal-and-Erythema Reaction.—The patients tested were: (*a*) F. O'B. had a history of an anaphylactic reaction to PG 5 years prior

TABLE X

*Skin Testing of Patients with Histories of Allergic Reactions to PG and Specific Inhibition of Wheal-and-Erythema Reaction with Di-BPO-EACA (Haptens)**

Patient	Antigens and hapten‡						
	HGG 50 μg.	HGG-BPO-(CSH), μg injected			Di-BPO-EACA (Hapten) 300 mμ moles (160 μg)	Mixture of HGG-BPO(CSH) (Ag) and Di-BPO-EACA (H)	
		0.4	4.0	40		Ag = 40 μg H = 300 mu moles	Ag = 4.0 μg H = 300 mu moles
M.O.§	0	1+	1+	2+	±	±	—
C.W.	±	2+	3+	—	±	—	1+
F.O'B.	0	1+	2+	—	±	—	0

* See Text for patients' histories. Antigens and haptens injected intradermally in 0.05 ml buffered saline. Readings at 15 minutes were graded: 0, 0–5 mm bleb no erythema; ±, 5–10 mm bleb slight or no erythema; 1+, 5 to 10 mm irregular wheal with 10 to 20 mm erythema; 2+, 11 to 15 mm irregular wheal with 20 to 30 mm erythema; 3+, 20 mm wheal with pseudopodia and 50 mm erythema. —, not tested. Five patients with no histories of PG allergic reactions gave 0 reaction to the antigen. The BPO-EACA hapten in the amount used did not inhibit wheal and erythema reaction given by 10 protein Nitrogen units of ragweed extract in two other patients with ragweed hayfever.

‡ HGG, native human γ-globulin; HGG-BPO (CSH)—see Table I; Di-BPO-EACA (0.4 per cent penamaldoyl preparation) converted to disodium salt by passage through Dowex-50 (Na^+) at pH 7 (11). Hapten and antigen were diluted in B.S. and sterilized by Seitz filtration.

§ M.O. exhibited a typical delyaed allergic reaction at 24 hours to the 40 μg dose of HGG-PBO (CSH).

to testing; (*b*) C. W., had a diffuse urticarial reaction to PG 8 days prior to the skin tests; (*c*) M. O., had 2 episodes of angioedema, occurring 2 days after PG, 8 years and 3 years prior to testing. The data in Table X show that the three patients reacted specifically to the HGG-BPO (CSH) antigen with typical wheal-and-erythema reactions. These reactions were strongly and specifically inhibited by Di-BPO-EACA hapten. Three other patients with histories of PG urticarial reactions occurring 2 to 5 years prior to skin testing gave negative reactions to HGG-BPO (CSH) and HGG-SS-BPE. One patient, M.O., exhibited a typical delayed (tuberculin) allergic reaction to 40 μg of HGG-BPO-

(CSH), as well as a wheal-and-erythema reaction. Hapten inhibition of wheal-and-erythema responses using the 2,4-dinitrophenyl system has been described previously (32).

DISCUSSION

Rabbits immunized with penicillin G (PG) or mixture of PG incubated with normal rabbit serum (PG-NRS) developed serum antibodies which reacted specifically with conjugates prepared by reaction of D-benzylpenicillenic acid (BPE) with various proteins and with poly-L-lysine. These conjugates contained a mixture of diastereomers of ϵ-N-(D-α-benzylpenicilloyl)-lysine (Di-BPO-Lys) groups, BPE-mixed disulfide (BPE-SS) groups (Table I), and appeared to contain also ϵ - N - (N^4 - (D - α - benzylpenicilloyl) - D - α - benzylpenamaldoyl)-lysine groups (Text-fig. 6).[8] The conjugates reacted with the rabbit pooled anti-PG and anti-(PG-NRS) sera both by precipitation (Tables III, IV, Text-figs. 7, 8, Fig. 1) and by PCA reaction in guinea pigs (Tables V, VI, Fig. 2). It is clear from the data on quantitative inhibition of precipitation by haptens (Table IV; Text-fig. 9), and the data on inhibition of PCA reaction by haptens (Tables V, VI; Fig. 2) that these antibodies were directed specifically against the diastereomeric BPO[9] antigenic determinant groups, which are bound in the conjugates predominantly as Di-BPO-Lys groups[5] (Table II). That the antibodies precipitated by the HGG-BPO (CSH) conjugate were directed against the BPO-Lys group is indicated also by the 40-fold greater effectiveness of BPO-EACA[1] over D-benzylpenicilloic acid as an inhibitor (Text-fig. 9). The greater effectiveness of Di-BPO-EACA over the α-BPO-EACA[1] in inhibiting both precitation (Text-Fig. 9) and PCA reactions (Table V, Fig. 2) given by the anti-PG sera with HGG-BPO (CSH) antigen demonstrates that the anti-penicillin antibodies were directed against the diastereomeric mixture of BPO-Lys groups.[9] The stereospecificity exhibited by the rabbit anti-BPO antibodies is in accord with the well known stereospecificity of anti-hapten antibodies (33, 34).

It is clear from the negative precipitation tests (Tables III, IV) and the

[8] The ϵ-N-(N^4-(BPO)-penamaldoyl)-lysine groups (Text-fig. 6) may not be formed *in vivo* where exceedingly low concentrations of PG (or BPE) react with tissue proteins.

[9] If, *in vivo*, PG reacts directly with lysine ϵ-amino groups of tissue proteins to form antigenic conjugates, the two asymmetric carbon atoms of the β-lactam ring (marked (*), Text-Fig. 1) are not involved and the resultant BPO-Lys groups would be α-diastereomers. If PG first rearranges to BPE which reacts with lysine ϵ-amino groups, the two asymmetric carbons of the β-lactam ring undergo epimerization and the resultant BPO-Lys groups would be formed as a mixture of the diastereomers. The finding that anti-PG antibodies were directed against the mixture of diastereomers of BPO-Lys is consistent with the view that PG reacts with tissue proteins *in vivo* through its intermediate BPE (1–3, 7). However, it is alternatively possible that PG may react directly to form the α-diastereomer of BPO-Lys which may secondarily undergo epimerization to form the mixture of diastereomers.

negative PCA reactions (Table VII) with the HGG-SS-D-penicillamine conjugate that the rabbit anti-penicillin sera contained no detectable antibodies directed against the D-penicillamine mixed disulfide group. Consistent with these data are the experiments of Josephson (6), who found that agglutination of "PG-coated" red blood cells by rabbit anti-PG sera was not inhibited by D,L-penicillamine. The data in Text-fig. 9, demonstrates that penamaldoyl-EACA[1] contained in the Di-BPO-EACA preparations did not inhibit precipitation of rabbit anti-PG sera by HGG-BPO (CSH), and indicates the absence of detectable antibodies directed against the N-(D-α-benzyl-penamaldoyl) group.[8]

Both specific precipitation (Table IV) and PCA reactions (Table VI) given by the pooled rabbit anti-PG and anti(PG-NRS) sera with HGG-SS-BPE antigen (containing 17 BPE-SS and 3.6 Di-BPO-Lys groups per mole) were completely inhibited by small quantities of Di-BPO-EACA. These results indicate that the Di-BPO groups contained in the HGG-SS-BPE conjugate were responsible for its specific reactions with the rabbit anti-penicillin sera. By these studies and also by agar gel double diffusion analysis (Ouchterlony) (Text-fig. 7; Fig. 1) no antibodies directed against the BPE-SS group were detected in the pooled rabbit anti-PG and anti-(PG-NRS) sera. The alternative possibility that anti-(Di-BPO) antibodies may cross-react with anti-(BPE-SS) antibodies would appear unlikely on the basis of the considerable structural differences between the two groups (Text-fig. 1). However, this possibility bears further study.[10]

The results of PCA analysis (with hapten inhibition of PCA) of one patient's serum (Table IX) and skin testing (with hapten inhibition of the wheal-and-erythema reactions) of several other patients (Table X) demonstrate that at least some patients with penicillin hypersensitivity of the immediate type develop serum antibodies and allergic skin reactivity specific for the BPO group. Further studies on the identities of the antigenic determinant groups responsible for penicillin hypersensitivity in human beings are currently in progress.

The above findings demonstrating the importance of the Di-BPO groups as antigenic determinants responsible for PG hypersensitivity of the immediate type[11] is consistant with the data in Table VIII showing the presence of non-

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[10] In early experiments a conjugated protein antigen containing only BPE-SS-Cys groups and capable of precipitating rabbit anti-(BPE-SS) serum did not precipitate the rabbit anti-PG serum.

[11] With regard to PG hypersensitivity of the contact dermatitis type, the BPE-SS-Cys and D-penicillamine-SS-Cys groups appear also to be important antigenic determinants (1). The importance of the mixed disulfide determinants in PG allergic contact dermatitis may be attributed to the reaction of PG (or BPE) with epidermal proteins which contain relatively large numbers of reactive cystine disulfide linkages (38).

dialyzable antigenic BPO-conjugates in the serum of a patient being treated with PG.

In the experiments described above, no antibodies directed against the BPE-SS and the D-penicillamine-SS groups were detected in either pooled rabbit anti-penicillin serum. Thus, either antigenic protein conjugates containing sufficient number of these groups were not formed *in vivo* by reaction of PG with tissue proteins, or these groups did not induce the formation of detectible antibodies. Both the BPE-SS and D-penicillamine-SS groups are capable of inducing antibody formation and hypersensitivity (1, 7, 35). Accordingly, and considering the small number of cystine disulfide linkages of serum proteins available for reaction (5), and the low therapeutic concentrations of PG (or BPE) achieved in tissue, it is possible that conjugates containing several mixed disulfide residues per mole may rarely be formed *in vivo*.

It is still possible, however, that some individuals may develop hypersensitivity to these groups. Further, in view of the numerous chemical reactions undergone by penicillin, still other derivative groups may prove to be antigenic determinants responsible for PG hypersensitivity.

It is generally agreed that simple chemicals induce hypersensitivity by combining irreversibly with tissue proteins to form the complete antigen (36, 37). In the cases of simple chemicals which are known to induce hypersensitivity and which do not undergo irreversible reactions with proteins, it was first suggested by Landsteiner (35) that these compounds may be converted to more reactive compounds *in vivo* which then react with tissue proteins. With regard to penicillin G, where no reaction of PG with proteins or low molecular weight protein model compounds had been demonstrated (1, 10 *d*, 39), it appears that a degradation product of PG, BPE, may be the protein-reactive pro-antigen responsible for the *in vivo* formation of the penicillin antigen (1–3, 7). Similarly, the ability of other low molecular weight chemical allergens such as aspirin and sulfonamides to induce hypersensitivity may be due to the *in vivo* formation of one or more reactive intermediate metabolites (*e.g.*, quinone derivatives) which may combine irreversibly with tissue proteins to form the complete antigen. It is to be noted that once a major antigent determinant group had been identified, and the proper complete antigens and univalent haptens prepared, the penicillin system was found to behave in many ways as do protein and conjugated-protein systems, *i.e.* exhibiting detectable precipitating and skin sensitizing antibodies, and hapten inhibition of specific reactions.

In this study, the general method of hapten inhibition as applied to precipitation reactions in aqueous (*cf.* reference 36) and gel media, to PCA reactions (*cf.* reference 31) and to wheal-and-erythema responses (*cf.* reference 32) demonstrated clearly the importance of the Di-BPO groups as antigenic determinants responsible for PG hypersensitivity of the immediate or anti-

body dependant type. Similar chemical and immunological studies may prove to be effective in identifying the antigenic determinants responsible for hypersensitivity to other allergenic drugs, such as aspirin and sulfonamides.

From the data presented here, it appears that conjugates containing several Di-BPO groups per mole may be important as antigenic reagents for diagnosis, prediction and investigation of penicillin hypersensitivity in human beings. Also, the hapten inhibition experiments described above suggest that univalent haptens such as Di-BPO-EACA may prove to be useful clinically as therapeutic agents to specifically inhibit antibody dependent penicillin allergic reactions in man.

SUMMARY

An excess of D-benzylpenicillenic acid (BPE) was reacted with human γ-globulin, human serum albumin, gelatin, and poly-L-lysine in aqueous solution buffered at pH 7.5–8.0. Under these conditions, BPE reacted predominantly with lysine ϵ-amino groups of the proteins to form the mixture of diastereomers of ϵ-N-(D-α-benzylpenicilloyl)-lysine groups (Di-BPO-Lys). BPE reacted also, but to a considerably smaller extent, with cystine disulfide linkages of human γ-globulin and human serum albumin to form D-benzylpenicillenic acid–cysteine mixed disulfide groups (BPE-SS-Cys). Conjugates containing large numbers of BPE or D-penicillamine mixed disulfide groups were prepared by reaction of BPE or D-penicillamine with thiolated human γ-globulin under mild oxidizing conditions.

Anti-penicillin antibodies were produced in rabbits by immunization with either potassium penicillin G (PG) or a preincubated mixture of PG with normal rabbit serum (PG-NRS) in complete Freund's adjuvant. Specific precipitation analyses in aqueous and gel media (Ouchterlony), PCA analyses, and specific inhibition of these reactions with haptens were carried out on the rabbit anti-PG and anti-(PG-NRS) sera, using the above conjugates as antigens. The anti-penicillin antibodies were found to be directed against the diastereomeric mixture of N-(D-α-benzylpenicilloyl) groups, predominantly the Di-BPO-Lys groups. By these techniques, no antibodies directed against the BPE-mixed disulfide or the D-penicillamine mixed disulfide groups were detected.

Three out of six patients with histories of allergic reactions to PG responded with wheal-and-erythema reactions to the N-(D-α-benzylpenicilloyl) (BPO) groups contained in BPE-human gamma globulin conjugate. Another such patient exhibited serum antibodies specific for the BPO group. One patient being treated with 25 gm per day of PG showed the presence of non-dialyzable antigenic BPO-conjugates in his serum.

These results demonstrate that the diastereomeric BPO groups (predomi-

nantly Di-BPO-Lys groups) are major antigenic determinant groups responsible for PG hypersensitivity in rabbits and human beings. The possible clinical usefulness of multivalent Di-BPO conjugates and univalent Di-BPO haptens is discussed.

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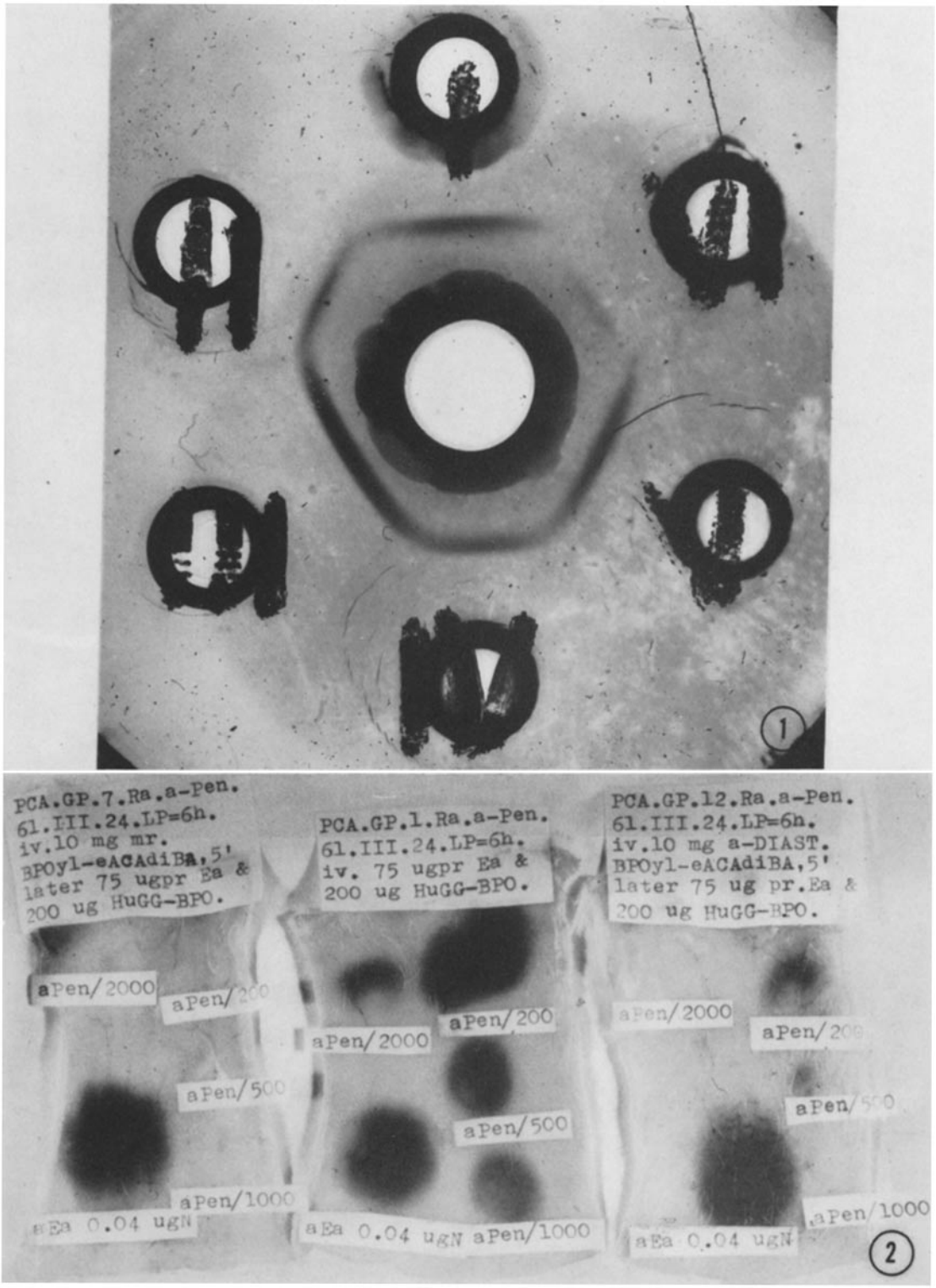
EXPLANATION OF PLATE 89

FIG. 1. Agar gel diffusion of the pooled Rabbit anti-PG Serum (see Text-fig. 1 *a*). Center well contained pooled rabbit anti-PG serum concentrated threefold. The peripheral wells contained: I and IV, HGG-BPO (CSH); II, HGG-BPO; III, PL-BPO; V, HSA-BPO all at 450 μg per ml in buffered saline; and VI, HGG-SS-BPE at 700 μg per ml in buffered saline. The plate was incubated at room temperature for 48 hours. See Table I for abbreviations of the conjugates.

FIG. 2. Specific hapten inhibition of penicillin hypersensitivity (PCA). PCA analysis of rabbit anti-penicillin serum and hapten inhibition of PCA reactions (See Table V). Each skin represents an individual guinea pig. The pooled rabbit anti-PG serum diluted in 0.15 M NaCl to 1:200, 1:500, 1:1000, 1:2000, and rabbit anti-ovalbumin (control) were injected intradermally (total volume = 0.10 ml). After 6 hours, the animals were challenged by intravenous injection of a mixture of 200 μg. HGG-BPO (CSH), 75 μg ovalbumin and 0.50 ml 1 per cent Evans blue (total volume = 1.0 ml). The animals were killed 12 minutes after challenge. The inhibited animals were injected intravenously with 10 mg of the hapten (skin on the right, α-BPO-EACA; skin on the left, Di-BPO-EACA) in 1.0 ml 0.15 M NaCl 5 minutes prior to injection of the antigen mixture.



(Levine and Ovary: Formation of penicillin antigen. III)