THE PREPARATION AND SOME PROPERTIES OF PENICILLENIC ACID DERIVATIVES RELEVANT TO PENICILLIN HYPERSENSITIVITY*·‡

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(Received for publication, November 22, 1961)

The basis for the suspicion that penicilloyl and penicillenate groups might be important antigenic determinants in human penicillin hypersensitivity has been developed elsewhere (1–3). Since the groups in question are simple organic structures it seemed very likely that the principles which apply to the elicitation of wheal-and-erythema responses by the dinitrophenyl determinant would also apply to the elicitation of skin responses with these penicillin derivatives. With the dinitrophenyl system, reagents containing 2 or more dinitrophenyl groups per molecule evoke skin responses but unifunctional substances, with 1 dinitrophenyl group per molecule, are specifically inhibitory (4). Dinitrophenyl-polylysine conjugates not only are effective in eliciting immediate skin responses but are not detectably immunogenic (5), and, in principle, the analogous penicilloyl-polylysine derivative should be the ideal substance for skin-testing human populations in order to determine whether the penicilloyl group is a determinant in human penicillin allergy.

Before evaluating the relative importance of the penicillenate[1] and penicilloyl determinants in penicillin sensitivity (3), it was necessary to establish the extent to which penicillenate-substituted conjugates are free of penicilloyl groups and, conversely, the extent to which penicilloyl conjugates are free of penicillenate residues. Moreover, it was essential to evaluate the possibility

* This work was supported, in part, by grants from the National Institute for Allergy and Infectious Diseases, United States Public Health Service (E-3231) and by a contract with the Research and Development Division, Office of the Surgeon General, recommended by the Armed Forces Epidemiological Board, Commission on Cutaneous Diseases (No. USDA-49-007-MD-695).

‡ Presented at the 34th annual meeting of the Central Society for Clinical Research, Chicago, November 3, 1961 (*J. Lab. and Clin. Inv.*, 1961, **58**, 948).

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[1] Benzylpenicillin is used throughout and all penicillin derivatives discussed are, therefore, benzyl-substituted on C^6 unless otherwise stated (see Fig. 1, *I*).

that substituents other than penicillenate and penicilloyl might be present in these conjugates. These questions, which are dealt with in the present paper, arise because penicillenate and penicilloyl conjugates are both prepared from penicillenic acid (1, 2, 6). Hence, the obvious difficulty in preparing conjugates which bear only a single kind of determinant is that most unmodified proteins have an abundance both of amino groups and sulfhydryl or disulfide groups. These offer opportunities during reaction with penicillenic acid for introduction of both penicilloyl substituents, attached to lysine residues, and penicillenate groups, attached to cysteine residues through direct attack on sulfhydryl or through initiation of disulfide interchanges.

The results given in the present paper indicate that polyfunctional penicillenate derivatives free of penicilloyl, and the converse, can be prepared. Although both classes of derivatives are contaminated by a third substituent of the penilloaldehyde-penamaldate type, this has not caused serious difficulty in evaluating the specificity of the immunologic responses of guinea pigs and rabbits to the penicilloyl and penicillenate groups.

Materials and Methods

The following reagents, and their sources, were used for preparative and analytical purposes: HSA,[2] Pentex Corp., Kankakee, Illinois; BγG, Armour and Co., Chicago; HγG, as poliomyelitis immune globulin, Pittman-Moore, Indianapolis; amberlite IRA 400, sodium arsenate, ammonium molybdate, sodium nitroprusside, and mercuric chloride (the latter 4 being "Fisher certified"), Fisher and Co., Fair Lawn, New Jersey; 2-mercaptoethanol, Evans blue, 2,4-dinitrophenylhydrazine, Eastman Organic Chemicals, Rochester, New York; D-penicillamine HCl, D-penicillaminic acid, L-cysteine·HCl, California Foundation for Biological Research, Los Angeles; *N*-acetyl-DL-homocysteine-thiolactone, Schwarz Bioresearch, Inc., Mt. Vernon, New York; *N*-ethylmaleimide, Mann Research Laboratories, New York; *p*-hydroxymercuribenzoate, Sigma Chemical Company, St. Louis; ε-aminocaproic acid, K. & K. Laboratories, Jamaica, New York; methylcellosolve, Heil Corporation, St. Louis; sephadex G-25, Pharmacia Laboratories, Inc., Rochester, Minnesota; methylethylketone, Merck and Co., Inc., Rahway, New Jersey.

We are grateful for the gifts of the following materials: potassium benzylpenicillin (1585 units per mg) and 6-aminopenicillanic acid, Chas. Pfizer and Co., Brooklyn, New York; polylysine-HCl with an average of 20 lysyl residues per molecule, Kremers-Urban Company, Milwaukee; 3,6 bis-(mercurimethyl)-dioxane, Dr. S. J. Singer, University of California at La Jolla, California; sodium decyl sulfate, Dr. F. Karush, University of Pennsylvania, Philadelphia.

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[2] Abbreviations: HSA, human serum albumin; BγG, bovine gamma globulin; HγG, human gamma globulin; penicillenate S–BγG and penicillenate S–HSA, penicillenate conjugates prepared by reaction of penicillenic acid with BγG and HSA previously treated with *N*-acetyl-DL-homocysteine-thiolactone in order to increase sulfhydryl content; MMD, 3,6-bis-(mercurimethyl)-dioxane; PNCE, penicillenic acid; bis-PNCE-MMD, 3,6-bis-(penicillenate-mercurimethyl)-dioxane; NES-PNCE, *S*-(*N*-ethylsuccinimidyl)-penicillenate; penicillamine S–HSA, the product obtained by coupling penicillamine to HSA previously thiolated by reaction with *N*-acetyl-DL-homocysteine-thiolactone. In the case of protein conjugates, subscripts correspond to the number of principal substituent groups per molecule; *e.g.*, penicillenate$_{50}$S–BγG is thiolated BγG with 50 penicillenate groups per molecule.

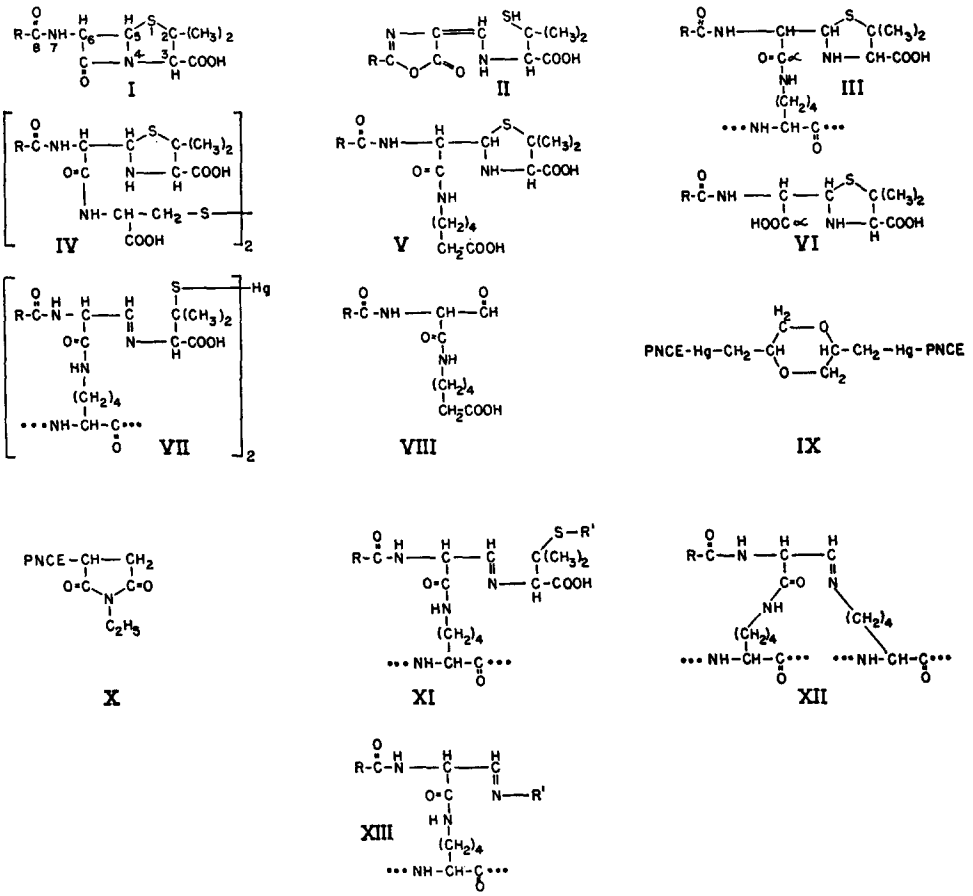

FIG. 1. Structural formulas of penicillin and penicillin derivatives (see footnote 1). *I.* Penicillin (R, benzyl), *II.* Penicillenic acid, *III.* α-(lysyl)-amide of penicilloic acid, *IV.* bis-penicilloyl-cystine, *V.* ε-penicilloyl-aminocaproic acid, *VI.* Penicilloic acid, *VII.* α-(lysyl)-amide of mercury penamaldate, *VIII.* ε-penilloaldehyde-aminocaproic acid, *IX.* 3,6-bis-(penicillenate-mercurimethyl)-dioxane; PNCE is penicillenic acid, *X.* *S*-(*N*-ethylsuccinimidyl)-penicillenate; PNCE is penicillenic acid, *XI.* α-(lysyl)-amide of penamaldate-mixed disulfide; R′ is any mercaptan, *XII.* Schiff's base conjugate of α-(lysyl)-amide of penilloaldehyde with lysine residue, *XIII.* Schiff's base conjugate of α-(lysyl)-amide of penilloaldehyde with a primary amine (=N—R′ represents the primary amine moiety).

The following were prepared by methods previously described: penicillenic acid (7), *p*-penicillenate-mercuribenzoate (2), penicillenic acid disulfide (2).

Penicilloyl-Protein Conjugates.—In aqueous solution at neutral or alkaline pH, penicillenic acid (Fig. 1, *II*) reacts with free amino groups forming the penicilloyl-α-amide (Fig. 1, *III* see references 1, 7, 8 *a*, 10 *a*). In the preparation of penicilloyl-HγG, 120 mg HγG were dissolved in 20 ml 0.15 M NaCl–0.1 M phosphate, pH 7.4, and 700 mg penicillenic acid in 4 ml 95 per cent ethanol were added in small increments with stirring over a 30 minute period. The pH was maintained between 7.5 and 8.5 by dropwise addition of 0.5 N NaOH. As judged

by the rate of fall of pH, the reaction was complete within several minutes after the last addition of penicillenic acid but the reaction mixture was allowed to stand at 4°C overnight. It was then passed through sephadex G-25 (washed with 0.15 M NaCl–0.01 M phosphate, pH 7.4), and the column effluent was monitored for protein with 10 per cent perchloric acid. The protein effluent was dialyzed for 4 days at 4°C against 0.15 M NaCl–0.01 M phosphate, pH 7.4, with IRA 400 (Cl^- form) in the outside solution the first 72 hours. The protein concentration was assayed by the biuret method. A similar procedure was used to prepare-penicilloyl-BγG.

The penicilloyl content of the protein derivatives was determined by titration with mercuric chloride (see below). The BγG and the HγG derivatives contained, respectively, 60 and 21 penicilloyl groups per molecule, assuming 160,000 as the molecular weight of the native proteins and correcting for the weight of the substituted groups.

Penicilloyl-Polylysine Conjugates.—Two different preparations of penicilloyl-polylysine were used in this study. In the first preparation (penicilloyl–polylysine A), 125 mg polylysine HCl were dissolved in 50 ml 0.1 M phosphate, pH 7.4, and 6.6 gm penicillenic acid in 20 ml 95 per cent ethanol were added over a 20 minute period with continuous stirring, the pH being maintained between 7.5 and 9.0 with 0.5 N NaOH. 30 minutes after all of the penicillenic acid had been added, the reaction mixture was concentrated *in vacuo* at 30°C to a volume of 20 ml on a rotatory evaporator, centrifuged to remove a small amount of insoluble material, and passed through a column of sephadex G-25 (25 gm, washed with 0.15 M NaCl–0.01 M phosphate, pH 7.4), the effluent being collected in 1 to 2 ml fractions. The fractions emerging with and close to the solvent front had strong absorbance at 287 mμ; these were combined and dialyzed (18/32 Visking sausage casing) for 4 days at 4°C against frequent changes of 0.001 M phosphate, pH 7.0. During the final 24 hours of dialysis the product was unchanged in respect to dry weight per ml and absorbance at 287 mμ.

A second preparation (penicilloyl–polylysine B) was prepared as above, except that during the initial reaction 0.002 M versene was present before addition of penicillenic acid, and the pH was maintained at 7.2–8.2; also, versene was used to prepare and elute the sephadex column and was present throughout dialysis (final concentration 0.0002 M). By the assay with $HgCl_2$ described below, it was estimated that the first polylysine preparation contained an average of 10 penicilloyl groups per molecule, and the second an average of 15 groups. Further characterization of these compounds is described under Results. After 2 months storage at 4°C the first preparation had retained 96 per cent of the original penicilloyl groups, and the second preparation had retained 95 per cent (estimated by mercuric chloride titration).

Penicilloyl-Cysteine and Bis-Penicilloyl-Cystine.—Penicilloyl-cysteine was prepared and isolated by the reaction of penicillin with cysteine in neutral anaerobic solution (8 *b*). A 3-fold molar excess of cysteine was used to minimize free penicilloate formation. Bis-penicilloyl-cystine (Fig. 1, *IV*) was prepared from penicilloyl-cysteine by air oxidation (18 to 20 hours) at pH 9.0 in 0.1 M sodium borate until the nitroprusside reaction in concentrated urea was negative. The reaction mixture was cooled to near 0°C, acidified to pH 1.8, and extracted with 2 volumes of chloroform-ether, 1:4 (*v/v*). The organic phase was kept in contact with anhydrous sodium sulfate for 15 to 30 minutes, decanted, and taken to dryness. The white powder obtained had a molar extinction coefficient of 42,000 on titration with mercuric chloride (see assay below), and in the arsenomolybdate assay (see below) gave a color yield which was 7 per cent of the value obtained with an equimolar quantity of penicilloic acid. *Analysis:*[3] Calculated for the disodium salt of $C_{38}S_4H_{48}O_{12}N_6$: C, 47.8; H, 5.04; N, 8.80; S, 13.42. Found (corrected for 5.26 per cent ash): C, 47.2; H, 5.42; N, 8.76; S, 13.9.

ϵ-*Penicilloyl-Aminocaproic Acid.*—10 ml 95 per cent ethanol was introduced anaerobically into an air-evacuated flask containing approximately 1.8 gm penicillenic acid. A deoxygenated

[3] Analyses of bis-penicilloyl-cystine and ϵ-penicilloyl-aminocaproate were performed by Micro-Tech Laboratories, Skokie, Illinois.

solution containing 3.7 gm ϵ-aminocaproic acid, 25 mmoles K_2HPO_4, 9 mmoles sodium borate, pH 9, 0.01 mmoles versene, and 20 ml 95 per cent ethanol in a total volume of 60 ml was added anaerobically. After 15 minutes the ethanol was removed on a rotatory evaporator at 30°C. The penicilloyl-aminocaproate (Fig. 1, *V*) was purified as follows: per ml of the aqueous solution there was added 500 mg ammonium sulfate and 2 ml methylethylketone. After acidification to pH 4.85 by dropwise addition of ice cold 6 N HCl, the aqueous phase was discarded. The organic phase was extracted with 0.5 volume 0.2 M Na acetate with enough 5 M NaOH being added to bring the final pH of the aqueous layer above 8.0. Extraction into methylethylketone and reextraction into sodium acetate was repeated 2 additional times. The distribution of penicilloyl groups (Hg assay, see below) between organic and aqueous phases at pH 4.85 was essentially constant on the last 2 passes (93 per cent in the organic phase). Following the third extraction cycle, the aqueous phase (pH 8.3) was carefully acidified to pH 3.0. A gummy precipitate formed and was washed twice with small volumes of water and air-dried. Yield, about 20 per cent. *Analysis.*[3] Calculated for monosodium salt of $C_{22}H_{31}N_3O_6S_1$: C, 54.0; H, 6.15; N, 8.60; S, 6.56. Found (corrected for 1.26 per cent ash): C, 55.5; H, 6.6; N, 8.54; S, 7.37.

In 0.05 M $NaHCO_3$, ϵ-penicilloyl-aminocaproate had no absorbance maximum at 322 mμ and from the absorbance at this wavelength its penicillenate content could not have exceeded 0.45 moles per cent. At 282 mμ, however, there was an absorption maximum, the absorbance per mg per ml amounting to only 1.7; on mercury titration a molar extinction coefficient of 21,300 was obtained.[4]

With the arsenomolybdate assay (see below), the color yield indicated that the ϵ-penicilloyl-aminocaproate could not have contained more than 4 moles per cent penicilloic acid.

Penicilloic Acid.—This derivative (Fig. 1, *VI*) was prepared by alkaline hydrolysis of penicillin (9) and also by reaction of penicillin with cupric sulfate in neutral solution with subsequent removal of copper with H_2S to yield the amorphous, gamma-rotatory penicilloic acid (8 *c*). M.p., 121° (Fisher-Johns microblock; uncorrected).

Mercury Titration for Penicilloyl Groups.—Woodward *et al.* have described the development of an intense ultraviolet absorption band, maximal at 282 mμ, when mercuric chloride is added to alcoholic solutions of penicilloates (8 *d*). Based on the suggestion of Levine (6) that this effect is applicable to analysis of penicilloyl-protein conjugates, the following procedure has been developed:

The reaction may be performed in dilute aqueous solution (0.05 M phosphate, pH 7.4, or 0.05 M bicarbonate-carbonate, pH 8–10). A preliminary titration to locate the end point is made first as follows: To 1.0 ml of solution in a cuvette containing 2 to 5×10^{-5} meq penicilloyl, a freshly prepared solution of $HgCl_2$ (2.58×10^{-4} M) is added in small increments of 0.01 to 0.02 ml, rapidly mixed, and absorbances read at 282 mμ about 1 and 3 minutes after each addition.[5, 6] Since excess $HgCl_2$ results in a decrease in absorbance beyond that expected

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[4] Penicilloate α-amides do not have an absorption maximum at 280 mμ. We ascribe the peak here to contamination with a small amount of either the corresponding penamaldate derivative of aminocaproic acid and/or the penamaldate derivative which has split out the penicillamine moiety to form a Schiff's base with excess amino-caproate (Fig. 1, *XIII*, where N=R′ is from ϵ-aminocaproate). These contaminants must, however, be present in only very small amounts since the absorbance of the mercury-treated solution gave the expected molar extinction coefficient (21,300 as compared with 18,000 and 20,900 reported in the literature for other α-penicilloates; see references 8 *d*, 8 *e*).

[5] *p*-hydroxymercuribenzoate produces a comparable absorption shift with penicilloyl compounds at 282 mμ.

[6] Titrations of penicilloyl-protein conjugates in 0.1 M sodium decyl sulfate containing one of the above buffers were nearly identical to those performed in the absence of the detergent.

on the basis of volume changes alone, it was necessary to approach the end point cautiously. A second titration is then performed using an initial volume of $HgCl_2$ which is exactly at, or 0.01 to 0.02 ml less than the previously estimated end point. Readings are taken at 1, 3, 5, and 7 minutes, and even later if absorbance continues to rise. Further 0.01 ml additions are made until it is clear that maximal change has been obtained. Duplicate determinations generally agree within 1 to 3 per cent. The reaction product of penicilloyl-lysyl residues with Hg is the mercury mercaptide of penamaldate (Fig. 1, *VII*). Using this reaction, the following molar extinction coefficients for the respective Hg-penamaldates have been obtained: 21,300 for ε-penicilloyl-aminocaproate; 42,000 for bis-penicilloyl-cystine; and 20,000 for penicilloic acid.[7] Published values for the molar extinction coefficient of the product formed by $HgCl_2$ reaction with penicilloyl α-amides are 18,000 and 20,900 (8 *d*, *e*).

Arsenomolybdic Acid Assay.—Penicilloic acid was assayed by the method of Pan (9).

ε-Penilloaldehyde-Aminocaproic Acid.—This derivative (Fig. 1, *VIII*) was prepared as follows: 100 mg penicilloyl-aminocaproic acid were dissolved in 4.3 ml 0.08 M K_2HPO_4, and 0.3 ml 1 N NaOH (final pH 7.2) and 32 mg $HgCl_2$ in 2.4 ml H_2O were added.[8] After 15 minutes the solution was chilled, acidified to pH 3, and after an additional 90 minutes the precipitate was discarded. A portion of the supernatant formed the expected insoluble product with 2,4-dinitrophenylhydrazine (8 *f*, 10 *b*). The latter derivative was crystallized from ethanol: m.p. 175° (Fisher-Johns microblock; uncorrected). The rest of the supernatant was extracted 4 times with an equal volume of ethyl acetate at 8°C. The extracts were combined, treated briefly with anhydrous sodium sulfate, and taken to dryness. Yield, 70 per cent. The gummy product had a molar extinction coefficient of 15,900 at 267 mμ in 0.1 M carbonate, pH 10.7, and gave a strongly positive Schiff's aldehyde reaction. These results are in agreement with observations made with other penilloaldehydes (8 *g*, 10 *c*).

Determination of Penilloaldehyde Groups.—The method of Lappin and Clark (11, 12) was modified as follows: 0.4 ml of protein (usually 3 mg per ml) or polylysine (1 to 2 mg per ml) was mixed with 0.4 ml 2,4-dinitrophenylhydrazine in 2 N HCl. After 30 minutes at 52°C, the solutions were chilled to near 0°C and diluted to 5 ml by the addition of ice cold alkaline methylcellosolve (11.8 gm potassium hydroxide in 20 ml water and 80 ml methylcellosolve) (13). The solutions were read immediately at 480 mμ. The values obtained with penicillenate and penicilloyl conjugates were compared with readings given by ε-penilloaldehyde-amino-caproate, both as a separate reaction mixture and when added as an internal standard. Un-

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[7] In the titration of these small molecular weight penicilloyl derivatives, the stoichiometry is 1 mole Hg^{++} for every 2 moles penicilloyl. In the titration of penicilloyl-proteins and -polylysines, relatively more $HgCl_2$ is required to produce a comparable rise in absorbance. If it is assumed that the molar extinction coefficient for Hg-penamaldates bound in amide linkage to proteins and polylysines is 21,000 to 22,000, then approximately 1 Hg is required per 1.6 penicilloyl groups. This effect might be ascribed to competition for Hg by groups other than penicilloyl. Native BγG does not have a significant effect on the titration of ε-penicilloyl-amino-caproate with $HgCl_2$. On the other hand, penicillenate and penamaldate groups present in protein and polylysine conjugates very likely do compete for Hg^{++}. Thus in comparing polylysine A and polylysine B (with and without 2-mercaptoethanol treatment), the degree of increase in absorbance at 282 mμ for a given increment of Hg^{++} varied inversely with the relative content of contaminating substituents of the penamaldate-penicillenate type (as judged by 322 and 282 mμ absorbance). Alternatively, steric interferences in proteins and polylysines might prevent the combination of Hg^{++} with 2 penicilloyl groups. In calculating the penicilloyl content of protein and polylysine we have used 21,300 as the molar extinction coefficient of the Hg-penamaldates.

[8] This was the equivalent amount of $HgCl_2$ based on the maximal increase of absorbance at 282 mμ.

conjugated proteins (HγG, BγG, and HSA) gave negligible blanks. An appropriate correction for the dinitrophenylhydrazine blank was made. When the solution was kept cold and read immediately, the molar absorbance for the reaction product obtained with ϵ-penilloaldehyde-aminocaproate was about 20,000.

Penicillenate-Protein Conjugates.—Methods used for preparation of thiolated proteins substituted with penicillenate residues have been reported previously (2). The procedure used for thiolation in the present study (a modification of the method of Singer *et al.*, 14) was adopted because of its greater simplicity and reproducibility. To a 5 per cent solution of protein in 0.15 M NaCl–0.01 M phosphate, pH 7.4, solid *N*-acetyl-DL-homocysteine-thiolactone was added in 12-fold molar excess with respect to lysyl residues of the protein. When the thiolactone had dissolved, the pH was raised by addition of 2 volumes 1.0 M sodium borate, pH 11.3. After 2 hours at room temperature, the solution was chilled, rapidly acidified to pH 4–5 with ice cold 6 N HCl (⌣ 0.1 volume), and 1/9 volume of ice cold 50 per cent trichloracetic acid was added. The precipitate was washed 3 times with ice cold 5 per cent TCA, and dissolved by the careful addition of 0.1 M K_2HPO_4 and 1 M NaOH to pH 6.5–6.8. The thiolated protein generally was titrated for sulfhydryl content and used for conjugation within 1 hour after preparation, but the rate of decrease of sulfhydryl groups in the thiolated protein was only 10 to 12 per cent in 48 hours when maintained in an oxygen-free solution at pH about 6.5. Protein recoveries were regularly 60 to 80 per cent based on absorbance at 280 mμ and on biuret analysis. The degree of thiolation as estimated by titration with bis-(mercurimethyl)-dioxane (14) or *p*-hydroxymercuribenzoate (15) was generally at or close to the theoretical maximum, that is, the number of lysyl residues of the protein.

In a typical conjugation of penicillenic acid to thiolated protein the following reagents were mixed rapidly in the order given: 70 gm urea; 20 ml 1 M phosphate–0.02 M versene, pH 7.1 (room temperature); 29 ml ice cold thiolated BγG, 11.1 mg protein per ml containing 60 SH groups per molecule; 18.5 mmoles penicillenic acid in 24 ml 95 per cent ethanol (ice cold); 80 ml 0.9 M borate–0.01 M versene, pH 9.1 (room temperature); and 3.5 ml 30 per cent H_2O_2 (ice cold). The pH of the reaction mixture fell quickly to between 7 and 8. After 1 hour at room temperature, the protein was precipitated by acidification to pH 4, washed repeatedly at −4°C with 95 per cent ethanol, passed through an IRA-400 column (Cl^- form) at room temperature, and dialyzed for 3 days at 4°C against 0.001 M phosphate, pH 7.0. Absorbance of the protein solution at 322 mμ remained essentially constant during the final 24 hours of dialysis. Based on biuret analysis and absorbance at 322 mμ the two thiolated BγG conjugates used contained 50 and 33 penicillenate groups per molecule, respectively, and the corresponding HSA conjugate contained 17 groups per molecule (2).

3,6 Bis-(Penicillenate-Mercurimethyl)-Dioxane.—This derivative (Fig. 1, *IX*) was prepared as follows: To a solution of 316 mg bis-mercurimethyldioxane in 350 ml 0.02 M K_2HPO_4, 380 mg penicillenic acid in 60 ml ethanol were added with continuous stirring over a 45 minute period. During the addition slight turbidity developed, and on standing in the cold for 4 days, a small amount of a crystalline material with an absorbance maximum at 355 mμ was formed (probably the mono-penicillenate-MMD product). Insoluble material was removed by centrifugation, and the supernatant was extracted with 1/5 volume chloroform at pH 4.0. The chloroform layer was removed and extracted 6 times with water to remove impurities and was then extracted with 1/2 volume 0.02 M K_2HPO_4 using enough 2 N NaOH to bring the pH of the aqueous phase to 7.5. The complete extraction cycle was repeated again and the final aqueous solution (pH 7.5) was acidified to 3.5. The precipitate that formed was washed 5 times with water and dried over $CaCl_2$ in a vacuum desiccator. The product had a molar extinction coefficient at 326 mμ of 43,400 in 0.15 M NaCl–0.01 M phosphate, pH 7.4. A second preparation was purified only by 4 cycles of precipitation at pH 3.5–4.0 and dissolution in 0.1 M $NaHCO_3$. After the third and fourth acid precipitation, portions were washed 5 times with water and dried over $CaCl_2$ for 72 hours; the molar extinction coefficients at 326 mμ were 43,300 and 43,000, respectively.

S-(N-EthylSuccinimidyl)-Penicillenate.—This derivative (Fig. 1, *X*) was prepared as follows: 0.4 mmoles *N*-ethylmaleimide and 0.4 mmoles penicillenic acid in 8 ml ethanol were added to 20 ml 0.1 M K_2HPO_4. After 90 minutes the reaction was complete as judged by stabilization of 322 mμ absorbance, which was 93 per cent of the theoretical value based on the original penicillenic acid content. The product was used without further modification.

D-Penicillamine S-HSA.—The method was similar to that employed for the preparation of penicillenate S-HSA. To 450 mg thiolated HSA (50 SH groups per molecule) in a volume of 30 ml 0.1 M phosphate, pH 6.6, were added 40 gm urea, 2.6 gm D-penicillamine in 10 ml water, 100 ml 0.01 M versene–0.9 M sodium borate, pH 9, and 4.5 ml 30 per cent H_2O_2. The product was dialyzed for 1 week against 0.001 M phosphate, pH 7.4.

D-Penicillamine Disulfide.—This was prepared as previously described and crystallized from ethanol-H_2O (8 *h*). M.p., 205° (dec.; Fisher-Johns microblock; uncorrected).

Chromatography.—Descending paper chromatography was performed with *n*-butanol:acetic acid:water (80:20:100) as developing solvent for 18 to 24 hours at 26°C. Penicillamine and its disulfide were detected by ninhydrin spray.

Preparation of Antisera.—Rabbits were immunized with penicillenate$_{50}$-S-BγG as described previously (2). With penicillenate S-BγG as precipitating antigen, the pooled sera contained 1.75 mg antibody protein per ml (2). With penicillenate S-HSA as precipitating antigen, the pool contained 1.62 mg antibody protein per ml.

Rabbit antiserum to the penicilloyl group was obtained by injecting rabbits with 5 mg penicilloyl-BγG in Freund's adjuvant (0.4 ml per foot-pad; see reference 2). After 1 month, an intravenous injection of 0.5 mg penicilloyl-BγG was given and selected sera obtained 5 days later were pooled. Analyses for antibody were performed with penicilloyl -BγG as the precipitating antigen. Precipitates were washed, dissolved in 0.25 M acetic acid, and absorbances determined at 322 and 280 mμ. In determining the antigen contribution to the 280 mμ absorbance, correction was made for the loss of absorbance at 322 and 280 mμ owing to acidity, the magnitude of the correction being based on the difference between absorbances of control solutions of the antigen in (*a*) 0.1 M sodium decyl sulfate–0.25 M acetic acid, and (*b*) 0.1 M sodium decyl sulfate–0.01 M phosphate, pH 7.4. By this procedure, the pooled anti-penicilloyl serum was estimated to contain 0.4 mg antibody protein per ml.

γ-globulin fractions of the above antisera were prepared with ammonium sulfate as described for human sera in the accompanying paper (3).

Passive Cutaneous Anaphylaxis.—The procedure followed was essentially that given by Ovary (16).

RESULTS AND DISCUSSION

The absorption spectrum of a representative preparation of a penicilloyl-protein conjugate is shown in Fig. 2. By contrast with absorption spectra of the native protein and the model compound bis-penicilloyl-cystine, it is clear that the conjugated proteins exhibit adventitious absorption at 322 mμ and at 280 mμ. The spectra of penicilloyl-protein conjugates are largely duplicated by penicilloyl-polylysines which have a shoulder at 310 to 330 mμ and an absorption maximum in the 280 to 290 mμ region (Fig. 3). Incubation of penicilloyl-polylysine with 2-mercaptoethanol results in a marked decrease in absorption above 300 mμ with the greatest change at 322 mμ where penicillenate absorbs maximally (Fig. 3). Since the mercaptan is known to cleave mixed disulfide bonds involving the penicillenate group, with subsequent degradation of penicillenic acid (2), we infer that the absorption around 320 mμ in penicilloyl-

proteins and in penicilloyl-polylysines is a consequence of contaminating penicillenate groups. In penicilloyl-proteins, penicillenate substituents can be ascribed to mixed-disulfide formation with cysteinyl residues. But in the penicilloyl-polylysines, an alternative explanation is required. Since the conditions used for preparation and purification of the polylysine conjugates virtually exclude the possibility of contamination with uncoupled penicillenic acid

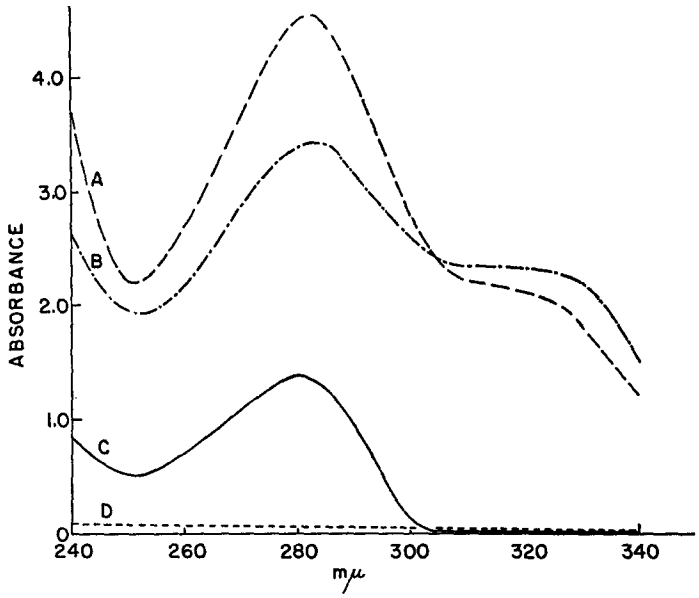

FIG. 2. Absorption spectra. Curves *A*, *B*, *C* are the absorption spectra of penicilloyl-HγG, penicilloyl-BγG, and BγG respectively, expressed as absorbance per milligram per milliliter. Curve *D* is the absorption spectrum of 5.8×10^{-5} M bis-penicilloyl-cystine. The molar concentration of penicilloyl groups is the same in curves *A* and *D*. The solvent in all instances was 0.15 M NaCl–0.01 M phosphate, pH 7.4.

disulfide, we infer that the penicillenate groups are substituted on the polylysines by mixed disulfide linkage with penamaldate groups which in turn are attached to the lysyl residues of the polyamino acid (Fig. 1, *XI*, where R′ is penicillenate). This latter structure could arise in one of two ways: (*a*) penicillenic disulfide in the original reaction mixture could directly acylate amino groups, or (*b*) penicilloyl groups attached to lysine side chains might undergo rearrangement leading to the formation of lysine-attached penamaldate groups which could then form mixed disulfides with penicillenic acid.[9] That the first

[9] In aqueous bicarbonate solution of penicillenic acid, a small absorption peak develops in the 280 to 290 mμ region, even in the absence of free amino groups. We presume this is due to penamaldate-penicillenate mixed disulfide, or to penamaldate disulfide.

mechanism proposed is feasible is indicated by the data shown in Fig. 4. When bis-PNCE-MMD[2] was mixed with a primary amine, there occurred a decrease in absorbance at 322 mμ, and a corresponding increase in absorbance at 280 mμ. Similar results were obtained on incubating penicillenic acid disulfide with ε-aminocaproic acid.[10] Additional evidence that penamaldate substituents occur in penicilloyl-protein conjugates was obtained in the following experiment which is based on the fact that at low pH penicillamine is cleaved from penamaldate (8 *d*, 10 *d*): A penicilloyl-protein conjugate was precipitated at pH 1,

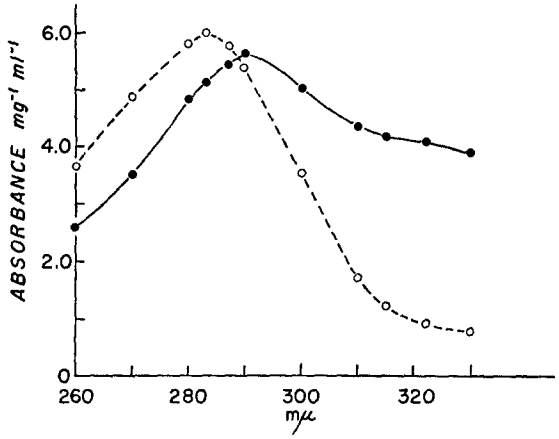

FIG. 3. Absorbance of penicilloyl–polylysine B before and after treatment with 2-mercaptoethanol. A solution of 23.5 mg penicilloyl–polylysine B in 5 ml 0.2 M $NaHCO_3$ was reacted with 0.2 ml mercaptoethanol under argon for 46 hours at room temperature. The solution was then passed through sephadex G-25 and dialyzed for 16 hours against water (18/32 Visking dialysis tubing). 22 mg polylysine were recovered. Absorbances were determined in 0.1 M phosphate, pH 7.2. Dashed line is absorption spectrum after 2 mercaptoethanol.

and the supernatant was subjected to chromatography using *n*-butanol–acetic acid–H_2O as developing solvent (see Materials and Methods). A ninhydrin-positive spot was observed which migrated with the same R_f as penicillamine disulfide.

The 280 mμ absorption peak in penicilloyl-polylysine, and the large increment in 280 mμ absorbance of the penicilloyl-proteins, can be readily accounted for by the presence of penamaldate substituents of various kinds: for example a 280 mμ absorbance band is expected for penamaldate (6 *d*), and also for pena-

[10] It should be stressed that a high concentration of primary amine was used and that the pH was elevated to 8. These conditions are present during the preparation of penicilloyl-polylysine and -protein conjugates. On the other hand, the absorption shift does not occur to a significant degree at pH 7.3, with a similar concentration of amine.

maldate combined through disulfide linkage with any of a variety of mercaptans (Fig. 1, *XI*, where R′ is any mercaptan), including penicillenate and penicillamine. The 280 mμ band could also arise from lysine-linked penamaldates whose sulfur is in various other states of oxidation; *e.g.*, $—SO_3$, $—SO_2H$, etc. (8 *i*).

In addition to penamaldate substituents, a number of other possibilities could account for the unexpectedly large absorbance at 280 mμ in penicilloyl

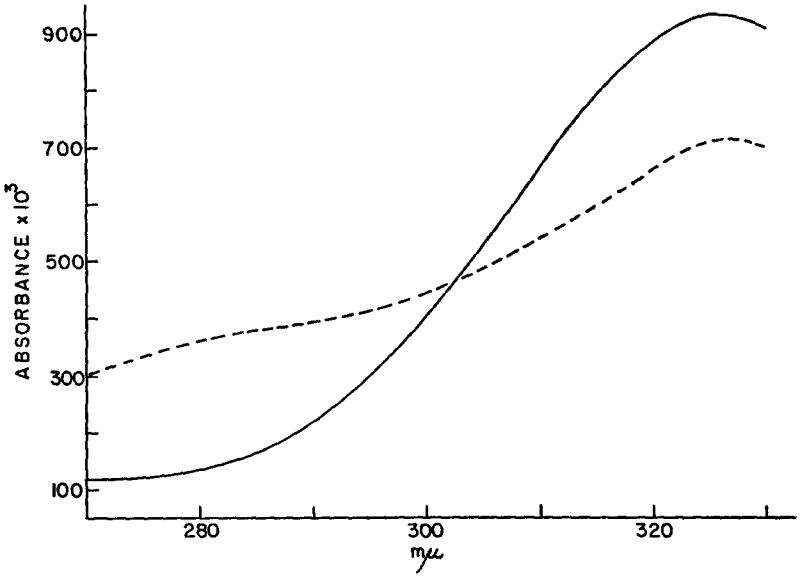

FIG. 4. Reaction of a mercury mercaptide of penicillenate with a primary amine. A solution consisting of 1.1×10^{-5} M bis-PNCE-MMD, 0.15 M ϵ-aminocaproic acid, and 0.075 M $NaHCO_3$, was kept at room temperature for 24 hours. The solid line is the absorption spectrum at zero time; the dotted line is the spectrum at 24 hours. The control solution, in which ϵ-aminocaproic acid was omitted, showed no significant spectral change during the 24 hour period.

conjugates. A particularly interesting possibility could arise from penilloaldehyde groups attached through their α-carboxyl to lysine residues, and combined additionally as Schiff's bases with neighboring amino groups (Fig. 1, *XII*); *e.g.*, the ϵ-amino groups of lysine residues in protein or polylysine. In agreement with older observations that penilloaldehydes couple with primary amines (8 *j*, 10 *e*), we have found that penilloaldehyde reacts in 0.1 M phosphate, pH 7.4, with ϵ-aminocaproic acid, with penicillamine disulfide, and with penicillaminic acid, in each case yielding derivatives with an absorption maximum at 280 mμ. The general formula of such structures is represented in Fig. 1, *XIII*, where =N—R′ could represent any primary amine.

Additional evidence that penicilloyl-polylysines, and presumably also peni-

cilloyl-proteins, contain substituents other than penicilloyl groups comes from a discrepancy between the number of such groups on a polylysine conjugate, as judged by mercuric chloride titration, and the substantially larger number of blocked amino groups as indicated by formol titration in the pH-stat: formol titration (17) revealed essentially no free amino groups in a polylysine preparation having, per molecule, a total of 20 lysine residues and only about 10 penicilloyl substituents. Moreover, penicilloyl-polylysines and penicilloyl-proteins gave positive reactions for aldehyde groups. A positive aldehyde reaction does not, however, distinguish between the various penamaldate and penilloaldehyde derivatives mentioned above because acidification of penamaldate ruptures the C=N bond, liberating aldehyde groups (and free penicillamine).

As expected from our interpretation of the 322 mμ absorption of penicilloyl-proteins, treatment of these conjugates with 2-mercaptoethanol substantially reduced the 322 mμ absorption and presumably eliminated contaminating penicillenate substituents.

While the penicilloyl-protein conjugates, untreated by 2-mercaptoethanol, are contaminated by penicillenate groups, the penicillenate-protein conjugates contain no penicilloyl groups demonstrable by mercuric chloride titration. Like the penicilloyl conjugates, however, the penicillenate conjugates have absorbances at 280 mμ which are in considerable excess of what is expected for the protein alone, or from a summation of the native protein's absorbance and the absorbance due to penicillenate substituents. These conjugates also give a positive reaction in the aldehyde assay, indicating substitution by groups of the penilloaldehyde-penamaldate type (see above). It will be recalled that penicillenic acid was coupled to protein after the latter had been thiolated; *i.e.*, substitution of lysyl residues with *N*-acetyl-DL-homocysteinyl groups. Thiolation was in all cases quite extensive. For example, with BγG, about 60 homocysteinyl groups were substituted as compared with a total of about 70 lysine residues per molecule. Nevertheless, some lysine side chains remained accessible for subsequent substitution by penicilloyl or penamaldate or penilloaldehyde groups. The absence of detectable penicilloyl by mercuric chloride titration may be ascribed to the fact that in the preparation of the penicillenate conjugates H_2O_2 was routinely present (8 *k*, 10 *f*). Thus, treatment of the model compound ε-penicilloyl-aminocaproic acid with H_2O_2 in bicarbonate leads to total loss of titratable penicilloyl groups with mercuric chloride, and to a 10-fold increase in absorption at 280 mμ, corresponding to the suggested rearrangement of the penicilloyl moiety to penamaldate.[11] Consistent also with

[11] Oxidation of free penicilloic acid with H_2O_2 at pH 8 does not produce a significant increase in absorbance at 280 mμ in contrast to the results with the ε-penicilloyl-aminocaproate and bis-penicilloyl-cystine. Evidently the amide bond at the α-carboxyl accounts for the stability of lysyl-bound penamaldate.

the latter possibility, is the fact that when H_2O_2-treated ϵ-penicilloyl-aminocaproate was acidified and subjected to chromatography (see Materials and Methods) a ninhydrin-positive spot with the R_f of penicillamine disulfide was obtained. In addition, adjustment of pH to 10.7 with 0.1 M carbonate after the acid treatment led to an absorbance peak at 267 mμ, corresponding to the tautomer of penilloaldehyde (8 *j*).

TABLE I

Evaluation of Reactivity of Various Penicilloyl and Penicillenate Derivatives with Rabbit Anti-Penicilloyl and Anti-Penicillenate Antisera by Means of Passive Cutaneous Anaphylaxis in the Guinea Pig

Antigen used to elicit skin response*	Anti-penicilloyl serum pool	Normal rabbit serum	Anti-penicillenate serum pool
Penicilloyl-HγG	4+	0	4+‡
HγG	0	0	0
Penicilloyl–polylysine A	4+	0	(±)
" " B	4+	0	0
" " B§	4+	0	0
bis-Penicilloyl-cystine	(±)	0	0
Penicillenate S–HSA	0‖	0	4+
HSA	0	0	0
Penicillamine S–HSA	0	0	4+
bis-Penicillenate-MMD	0	0	(±)
p-Penicillenate-mercuribenzoate	0	0	0

* In each guinea pig 5 skin sites were prepared, duplicate sites with each of the antiserum pools and 1 site with normal serum. 2 guinea pigs were tested with each antigen. Where a discrepancy existed, additional guinea pigs were tested (penicillenate S–HSA, 4 animals; bis-penicilloyl-cystine, 3 animals; bis-penicillenate-MMD, 3 animals). Values in parentheses are approximate averages, the individual responses varying from 0 to 1+.

‡ Although 4+ by the criteria used (16), these reactions were definitely less strong than the responses at the site prepared with the anti-penicilloyl serum pool.

§ Treated with 2-mercaptoethanol to remove penicillenate groups.

‖ In 1 of the 4 animals tested these sites gave a 2+ response.

Immunologic evaluation of the penicilloyl- and penicillenate-protein conjugates with reference to their distinctive specificities are summarized in Table I and in Fig. 5. In keeping with the chemical evidence that penicilloyl-HγG contains approximately 12 to 15 penicillenate groups (in addition to 21 penicilloyl groups) this conjugate evokes strong reactions in skin sites prepared with either anti-penicilloyl rabbit sera or rabbit antisera to penicillenate-protein conjugates. Likewise consistent with the chemical evidence that penicilloyl groups are absent from penicillenate conjugates are the observations that skin sites prepared with anti-penicilloyl sera fail to react to penicillenate-protein conjugates, and sites prepared with anti-penicille-

nate sera fail to react to penicilloyl–polylysine B. However, penicilloyl–polylysine A, which contained approximately 3 to 4 penicillenate groups per molecule, did evoke a weak response in sites prepared with anti-penicillenate serum. The failure of penicilloyl–polylysine B to react with anti-penicillenate

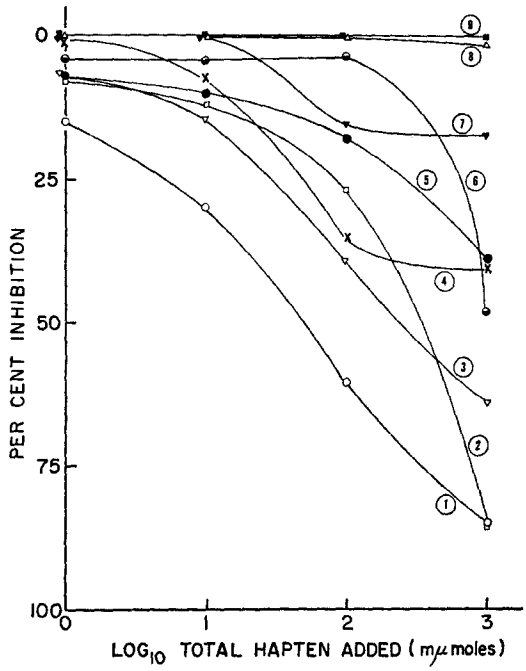

FIG. 5. Inhibition of precipitation of rabbit anti-penicilloyl antidoby with haptens. Precipitin reactions were carried out in the region of slight antigen excess using 0.4 ml of a γ-globulin fraction prepared from rabbit anti-penicilloyl serum and 100 μg penicilloyl-BγG in a total volume of 1.0 ml. Control precipitates (haptens absent) contained 290 μg antibody protein. Curves representing inhibition by various haptens are as follows: *1*, ε-penicilloyl-aminocaproate; *2*, ε-penilloaldehyde-aminocaproate; *3*, bis-penicilloyl-cystine; *4*, penicillin; *5*, *p*-penicillenate-mercuribenzoate; *6*, penicilloic acid; *7*, 6-aminopenicillanic acid; *8*, penicillamine disulfide; *9*, penicillamine.

50 mμmoles ε-penicilloyl-aminocaproate in competition with 38 mμmoles penicilloyl groups as penicilloyl-BγG inhibited precipitation to the extent of 50 per cent.

serum can be ascribed to the conjugate's having only 1 to 2 penicillenate groups per molecule. As expected, the mercaptoethanol-treated polylysine B (which had virtually no 322 mμ absorption) failed also to react with skin sites prepared with anti-penicillenate sera (Table I).

Since substituents of the penilloaldehyde-penamaldate type are present in both immunizing antigens (penicilloyl-BγG and penicillenate S–BγG) and

also in the eliciting antigens (penicilloyl-polylysine and penicillenate S–HSA) considerable cross-reactivity might have been anticipated. That this was not the case indicates that the penamaldate-penilloaldehyde groups are relatively inert immunologically, at least in the presence of a larger number of penicillenate or penicilloyl substituents on the same molecule. An alternative possibility is that the groups responsible for 280 mμ absorption in penicilloyl-polylysines are different from those in penicillenate-protein conjugates.

The reactions evoked by penicillamine S–HSA in sites prepared with anti-penicillenate serum may seem at first to raise some question as to whether the specificity of the response is directed at all against the penicillenate group. Actually, however, the reaction between penicillamine S–HSA and anti-penicillenate sera seems to be a consequence of the homocysteinyl groups of the thiolated proteins used for immunization and elicitation. Thus, precipitation of anti-penicillenate sera by penicillenate S–HSA was inhibited 65 per cent with 10^{-3} M *p*-penicillenate-mercuribenzoate, but was not inhibited at all by penicillamine or penicillamine disulfide. Moreover, anti-penicillenate serum was precipitated specifically, to a slight extent, with penicillamine S–HSA, and this precipitation could be substantially inhibited by *N*-acetyl-DL-homocysteine, but not at all by penicillamine. Hence the skin reaction evoked by penicillamine S–HSA can be ascribed to the fact that, in part, the specificity of the anti-penicillenate serum pool is directed against *N*-acetyl-DL-homocysteinyl groups (most likely the D-enantiomorph) which are abundant in the immunizing antigen. In large measure, we ascribe the decreased specificity for penicillenate in the present antisera, as compared with results described previously (2), to the fact that the immunizing antigen used in the present work (penicillenate S–Bγ G) was much more extensively thiolated than previously.

In regard to the serum prepared against penicilloyl-proteins, hapten inhibition data, summarized in Fig. 5, indicates that in spite of the conjugates having substituents other than penicilloyl residues, the specificity of these sera is directed almost entirely against penicilloyl. Thus, ε-penicilloyl-aminocaproate inhibited precipitation of anti-penicilloyl serum by penicilloyl-Bγ G almost completely. The effectiveness of bis-penicilloyl-cystine and the penilloaldehyde derivative of aminocaproate as inhibitory haptens is probably due to cross-reactivity of these haptens with antibodies formed against penicilloyl-lysyl groups, rather than to the presence of antibodies primarily specific for these radicals. But antibody homologous for penilloaldehyde groups cannot be excluded. The relative effectiveness of ε-penicilloyl-aminocaproate, bis-penicilloyl-cystine, and ε-penillo-aldehyde-aminocaproate as inhibitors of precipitation as contrasted with penicilloic acid stresses the importance of the alkyl chain of lysine and the amide linkage at the α-carboxyl in the binding of penicilloyl-proteins by antibody.

From the chemical evidence presented, there clearly are a variety of determi-

nants present in the conjugates which are predominantly penicilloyl-substituted and in those which are predominantly penicillenate-substituted. Nevertheless, these conjugates appear to be sufficiently distinctive as antigens to provide the means for preliminary evaluation of the significance of penicilloyl and penicillenate groups in hypersensitivity to penicillin. This evaluation is the objective of the following paper (3).

SUMMARY

A number of penicillenate and penicilloyl derivatives potentially useful in the study of penicillin hypersensitivity have been prepared and some of their properties described.

We wish to thank Mr. Walter Gray and Mr. Richard Pinkston for competent technical assistance in certain phases of this study.

Note Added in Proof.—Since submission of this and the accompanying papers (3, 5), Levine and Ovary (*J. Exp. Med.*, 1961, **114,** 875) have reported that in rabbits injected with benzylpenicillin antibodies specific for the penicilloyl group appeared but antibodies specific for the penicillenate group were not demonstrated (see also reference 6).

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