

SOME IMMUNOCHEMICAL PROPERTIES OF PENICILLINIC ACID

AN ANTIGENIC DETERMINANT DERIVED FROM PENICILLIN*

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Penicillin hypersensitivity in man is recognized as a clinical problem of considerable magnitude (1). Although this problem has received much attention, the antigenic determinant(s) responsible are not known. The identification of the determinant(s) is important for several reasons. First, it could permit the preparation of reliable reagents for identifying hypersensitive individuals. Second, it could make possible the study of the biology of hypersensitivity in a large reservoir of human beings who are sensitive to a simple organic molecule. Third, it could lead to the preparation of highly purified human antibody, specifically adapted to a defined structure, in amounts adequate for structural studies of human γ -globulin.

On the basis of general experience with antibody induction by low molecular weight compounds, it is necessary to assume that the actual sensitizer in penicillin hypersensitivity is capable of reacting with proteins to form stable conjugates (2). Since there is no indication that penicillin can react with proteins in the required manner, we have assumed that the sensitizing agent in penicillin hypersensitivity is not the penicillin molecule itself, but a metabolite or a derivative. It may be anticipated that such a hypothetical substance would have the following three properties: (a) form from penicillin under relatively mild conditions (such as physiological states); (b) be able to conjugate with proteins by means of covalent linkages; (c) contain a potentially antigenic configuration. Of the many known breakdown products of penicillin, the one

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which seems to us to come closest to meeting these requirements is penicillenic acid. Penicillenic acid forms readily in aqueous solutions of penicillin under mild conditions (3 *a*). In addition, this derivative has a free sulfhydryl group, suggesting possibilities for conjugation with many proteins. Finally, its structure is closely related to 2-phenyl-4 ethoxy-methylene-oxazolone. The latter was shown 14 years ago by Gell, Harington, and Rivers to be a potent inducer of serum antibodies in rabbits (4).

In the present work, the conversion of penicillin to penicillenic acid has been studied, and stable penicillenic acid-protein conjugates have been prepared by means of mixed disulfide linkages with several proteins. The conjugated proteins induce in guinea pigs and rabbits allergic skin reactivity and substantial amounts of antibodies, specifically directed to the penicillenic acid group. The results of these preliminary studies suggest the potential importance of penicillenic acid as a sensitizer and as an antigenic determinant in penicillin hypersensitivity in human beings. It must be emphasized, however, that direct evidence bearing on these possibilities is not yet available.

EXPERIMENTAL¹

Occurrence of Penicillenic Acid (PNCE) in Aqueous Solutions of Benzylpenicillin.—Penicillenic acid (PNCE) forms spontaneously in aqueous solutions of penicillin² by a rearrangement which involves splitting of the β -lactam ring, formation of an oxazolone ring, and appearance of a free sulfhydryl group (Fig. 1) (3 *b*). PNCE is readily detected and measured by virtue of its strong absorbance at 320 $m\mu$ (Fig. 2). This absorption band seems to be quite specific for the oxazolone ring with a double bond in the 4- position, as none of the other known degradation products of penicillin exhibits significant ultraviolet absorption at this wavelength (3 *c*).

¹ Potassium benzylpenicillin (1585 units/mg.), allythiomethylpenicillin (penicillin O), and sodium 6-aminopenicillanic acid were generously provided by Dr. B. Sobin and Dr. D. Iezzoni of Chas. Pfizer and Co., Brooklyn, New York. Mercaptoethylamine HCl and α -monothio-glycerol were kindly furnished by Evans Chemetics, Inc., New York. Glutathione and mercaptoethylamine were obtained from Nutritional Biochemical Co., Cleveland, Ohio, and mercaptoethanol and thioglycolic acid from Eastman Kodak Co., Rochester, New York. L-Cysteine HCl was a product of Pfanstiehl Co., Waukegan, Illinois, and *p*-chloromercuribenzoate, Na salt, was from Sigma Chemical Co., St. Louis, Missouri. Schwarz laboratories, Mt. Vernon, New York, furnished thiolated gelatin (thiogel) and *N*-acetyl homocysteine thiolactone.

The proteins used, and their sources were: bovine serum albumin and bovine γ -globulin (Armour and Co., Kankakee, Illinois); human serum albumin (Squibb and Sons, New York); human γ -globulin in the form of "poliomyelitis immune human γ -globulin" (Lederle Laboratories, Pearl River, New York).

² Unless stated otherwise, penicillin refers to benzylpenicillin, and all derivatives of penicillin are derivatives of benzylpenicillin. Abbreviations used for penicillin and some of its derivatives are given in the legend of Fig. 1.

As shown in Figs. 3 *a* and 3 *b*, the rate of appearance of absorbance at 320 $m\mu$ in aqueous solutions of penicillin is dependent upon pH and temperature. The formation of PNCE occurs at neutral pH, but is much more pronounced in the acid range. No 320 $m\mu$ absorbance appears at pH 9.

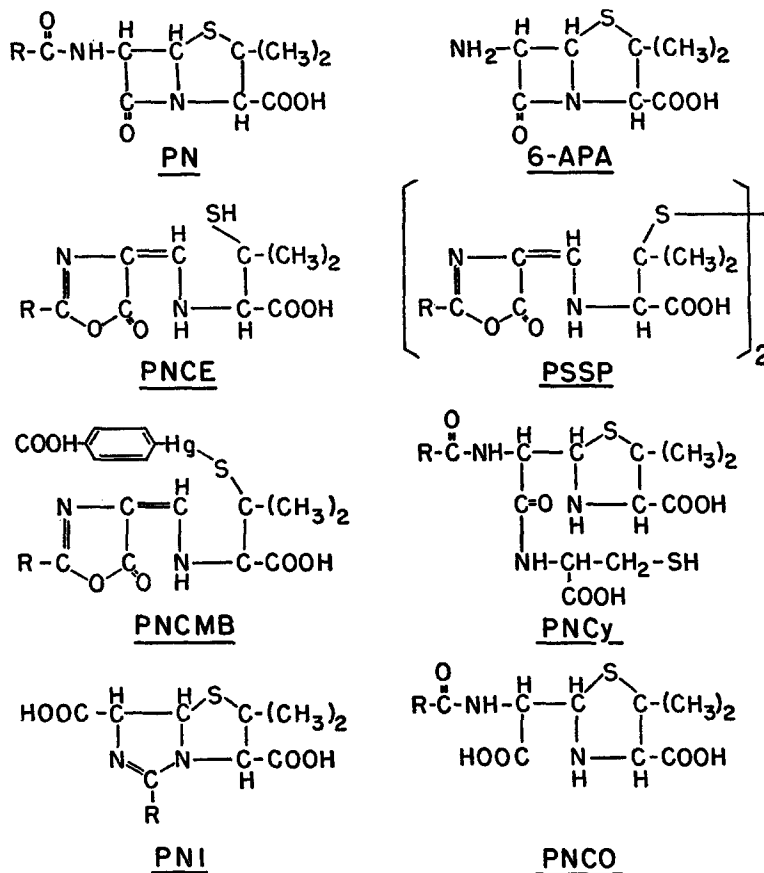


FIG. 1. Structural formulas of penicillin and penicillin derivatives.

PN; benzylpenicillin (R, benzyl)
 [PNO, allylthiomethylpenicillin (R,
 $\text{CH}_2=\text{CH}-\text{S}-\text{CH}_2^-$)]
 6-APA, 6-aminopenicillanic acid
 PNCE, penicillenic acid
 PSSP, penicillenic acid disulfide

PNCMB, *p*-chloromercuribenzoate derivative
 of penicillenic acid
 PNCy, cysteine derivative of penicillin
 PNI, penillic acid
 PNCO, penicilloic acid

The initial rate of formation of PNCE from penicillin at 25° and pH 4 (0.1 M acetate) was linear with initial penicillin concentration, in the range of 1×10^{-2} to 1×10^{-4} M (0.4 $m\mu$ -moles PNCE/ μ mole penicillin/ml/min.). Under these conditions, however, the increase in 320 $m\mu$ absorbance is linear with time only for the first 15 to 20 minutes. Thereafter, the rate

decreases, suggesting PNCE instability. When PNCE (see below for preparation) is dissolved in water, it decays rapidly. At pH 7.4, its half-life is about 60 minutes, but the decay is much faster at extreme acid and alkaline pH values (Fig. 4).

PNCE is an unstable intermediate in the degradation of penicillin to penillic and penicilloic acids (Fig. 1). The main degradation product of penicillin at acid pH is penillic acid (3 *d*, 5). Needle-like crystals appeared in concentrated ethanol solutions of PNCE on standing in the cold for several days. These crystals had the same melting point (188–189°) as reported for authentic penillic acid (3 *e*). At alkaline pH, on the other hand, penicillin and PNCE are both rapidly converted to penicilloic acid (3 *f*, 3 *g*). Long standing alkaline aqueous solutions of penicillin, as well as slightly alkaline PNCE solutions, exhibit a shift of their end absorption towards lower wavelengths, as described for benzylpenicilloic acid (3 *c*). Stronger alkaline

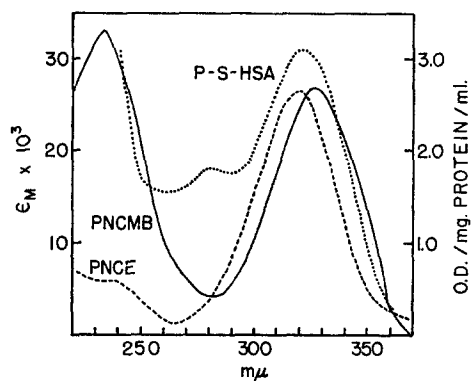


FIG. 2. Ultraviolet absorption spectra. Absorption spectra expressed as molecular extinction coefficient for penicillenic acid (PNCE, in 95 per cent ethanol) and for the mercuribenzoate mercaptide of penicillenic acid (PNCMB, in 0.1 M KHCO₃) and as absorbance (O. D./mg. protein/ml.) for P-S-HSA conjugate (in 0.25 M acetic acid). See legend of Table III for abbreviations of protein conjugates.

hydrolysis converts PNCE to penicillamine and 2-benzyl-4 hydroxymethylene-5(4)-oxazolone (3 *h*).

The rate of conversion of penicillin to PNCE, at pH 4.0 and 25°, is estimated from the initial rate of formation of 320 mμ absorbance, to account for a loss of 24 per cent of the penicillin in 12 hours. This rate is much slower than that at which penicillin solutions lose antibiotic activity under these conditions (50 per cent loss in 12 hours, see reference 6). Accordingly, it is probable that PNCE is not an obligatory intermediate in the degradation of penicillin.

While the degradation of PNCE to penillic and penicilloic acid leads to a loss of 320 mμ absorbance, the simultaneous formation of penicillenic acid disulfide (PSSP, Fig. 1), which retains the 320 mμ absorption peak, tends to stabilize optical density at this wavelength. The formation of PSSP, and its stability are discussed below.

The concentration of PNCE in aqueous solutions of penicillin depends, therefore, on the outcome of many opposing reactions. A qualitative correlation between these reactions and the over-all time course of appearance and disappearance of 320 mμ absorbance in penicillin solutions may be obtained by comparing Table I with Figs. 3 *a* and 3 *b*.

*Preparation of Penicillenic Acid (PNCE)*³.—PNCE was prepared by Levine's procedure (7) which constitutes a useful modification of the method of Carpenter, Turner, and du Vigneaud (5).⁴ The method is based on the degradation of benzylpenicillin to the mercury mercaptide of benzylpenicillenic acid by mercuric chloride, regeneration of the mercaptan by H₂S, and extraction into cold benzene. Lyophilization of the benzene extract yielded an amorphous white powder containing 90 to 92 per cent penicillenic acid (as calculated from $E_M = 26,600$ at 320 m μ) (5). Yields ranged from 20 to 25 per cent but decreased if the benzene extract was not promptly processed and lyophilized, or if insufficient volumes of benzene were used.

TABLE I
Influence of Various Factors on Appearance and Disappearance of a 320 m μ Absorption Band in Aqueous Solutions of Benzylpenicillin

pH	Conversion of penicillin to PNCE*	Decay of PNCE*	Formation of penicillenic disulfide (PSSP) ^{†,§}	Stability of PSSP [§]	Direct conversion of penicillin to other compounds*
3.0 and below	Fast	Very fast	None detected	Unstable	Very fast
4.6	Fast	Fast	Very slow	Stable	Fast
7.4	Slow	Slow	Moderate	Stable	Slow¶
9.0	None detected	Fast¶	Fast**	Stable	Very fast¶

* Rate increases with temperature.

† Judged from the stabilization level of the 320 m μ reading.

§ Influence of temperature not determined.

|| Degradation mainly to penillic acid (3d, 5).

¶ Degradation mainly to penicilloic acid (3g).

** Only in presence of 8 M urea and hydrogen peroxide.

Lyophilized PNCE was stable for several weeks in a desiccator at -15° . At room temperature in a desiccator, the PNCE content of the amorphous powder decreased from 92 per cent to 80 per cent over a period of 2 weeks. PNCE is very soluble in ethanol, acetone, chloroform, ether, and amyl acetate. It is sparingly soluble in water, maximal solubility being about 1 mg./ml. at pH 4.0, 2.5 mg./ml. at pH 7.4, and 5 mg./ml. at pH 9.0. Ethanol solutions are stable in the cold up to several hours, but the 320 m μ absorbance decreases at room tempera-

³ Preliminary attempts to prepare penicillenic acid were based on the spontaneous degradation of penicillin to penicillenic acid in water or in chloroform (3a). After 4 to 6 days' incubation of penicillin in 0.44 M acetate, pH 4.6, and Cu₂SO₄ (3.3×10^{-5} M) (17), at room temperature, the 320 m μ absorbance corresponded to 40 to 50 per cent of initial penicillin, assuming that the molecular extinction coefficient for PNCE at this wavelength is 26,600 (5). By heating penicillin solutions in a boiling water bath for periods up to 10 minutes (18), the rate of conversion to PNCE was accelerated, but the final yield was lower (~7 to 10 per cent). Concentration and stabilization of the 320 m μ absorbing material were achieved by acidifying the aqueous solutions to pH 2.5, extracting with chloroform, and dissolving in ethanol the residue obtained by drying the chloroform extract *in vacuo*.

⁴ We are grateful to Dr. Bernard B. Levine for communication of his method prior to publication (7).

ture to 70 to 75 per cent of its initial value in 24 hours. PNCE is quite unstable in water, depending on pH, temperature, and ethanol concentration of the solution.

Reaction of Penicillenic Acid (PNCE) with Simple Mercaptans.—The SH group of PNCE, with its potentialities for forming disulfides and mercaptides, was exploited in the preparation of haptens and protein conjugates. Aside from convenience, substitution of the SH group had the important advantage that it stabilizes the oxazolone structure. Accordingly, the 320 $m\mu$ absorption

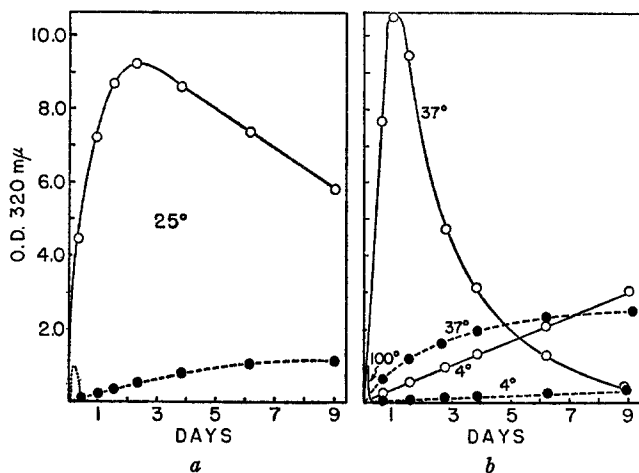


FIG. 3 *a*. Effect of pH on conversion of benzylpenicillin to PNCE. Benzylpenicillin at an initial concentration of 0.01 M was incubated at room temperature ($\sim 25^\circ$), in 0.1 M acetate, pH 3.0 (.....), or in 0.1 M acetate, pH 4.6 (O—O), or in 0.1 M phosphate, pH 7.4 (●---●).

FIG. 3 *b*. Effect of temperature on conversion of benzylpenicillin to PNCE. Benzylpenicillin at an initial concentration of 0.01 M was incubated at the indicated temperatures, at pH 4.6 (O—O) and at pH 7.4 (●---●).

band continued to be useful analytically in studying the reactivity of the PNCE sulfhydryl and the stability of PNCE disulfides and mercaptides.

The nitroprusside reaction of PNCE in aqueous solution is weaker than expected, possibly because of hydrogen bonding of the sulfhydryl (3 *a*), or because of the presence of penicillenic acid disulfide (PSSP) in such solutions. That there does occur a significant degree of hydrogen bonding was indicated by the increased nitroprusside reaction in 8 M urea. A similar effect is known in the case of some other mercaptans; *e.g.*, glutathione (8).

As noted previously, PNCE in aqueous solution at pH 7, decays rapidly for the first 60 minutes, but thereafter the 320 $m\mu$ absorbance tends to become stable. We ascribe the stabilization to penicillenic acid disulfide (PSSP) formation for two reasons: (*a*) the nitroprusside reaction of the stabilized solution is negligible, but becomes positive on addition of cyanide or of a small known

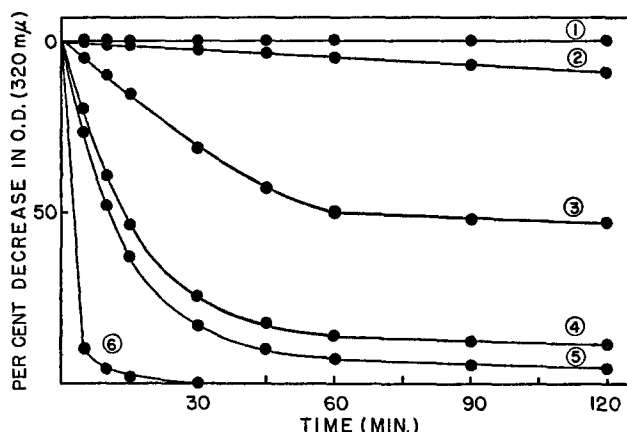


FIG. 4. Influence of pH on PNCE decay. A stock solution of PNCE in 95 per cent ethanol was diluted to a final PNCE concentration 4 to 7×10^{-5} M in 95 per cent ethanol (curve 1) and in 30 per cent ethanol, 70 per cent 0.01 M phosphate, pH 7.4 (curve 2). The same stock solution was also diluted (to make a final concentration of 4 to 7×10^{-5} M PNCE and 1 per cent ethanol) in the following aqueous buffers: 0.01 M phosphate, pH 7.4 (curve 3); 0.01 M borate, pH 9.0 (curve 4); 0.01 M acetate, pH 4.6 (curve 5) and pH 3.0 (curve 6). All solutions were incubated at 25° , except for curve 1 (4°).

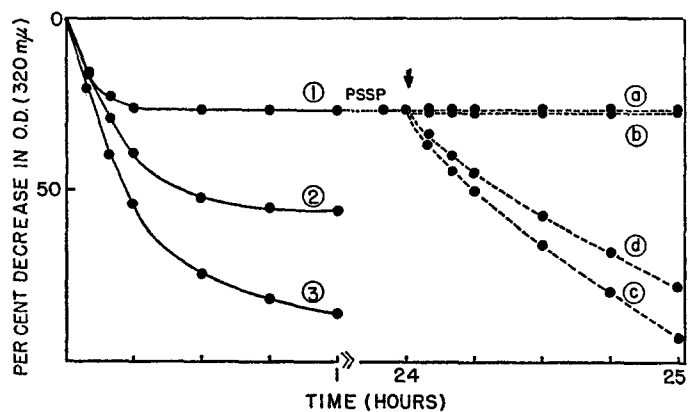


FIG. 5. Formation of penicillenic acid disulfide (PSSP). A stock solution of PNCE in 95 per cent ethanol was diluted to a final concentration of 7×10^{-5} M PNCE, 1 per cent ethanol, in the following solvents: 10^{-2} M borate, pH 9, 10^{-3} M versene, 8 M urea, 1.5 M H_2O_2 (curve 1); 10^{-2} M borate, pH 9, 10^{-3} M versene, 8 M urea (curve 2); 10^{-2} M borate, 10^{-3} M versene (curve 3). An aliquot of the original stock solution was diluted in 95 per cent ethanol and kept at 4° for reference purposes. The solution corresponding to curve 1 remained optically stable for 1 to 24 hours at room temperature and consisted, presumably, of PSSP. At 24 hours (see arrow) to an aliquot of this solution thioglycolic acid in excess was added curve *a*). Another aliquot had its pH adjusted to 4.6 (curve *b*) with HCl to match the pH of the thioglycolic acid-treated sample. Other aliquots had pH adjusted to 7.4 (curve *a*) and to 2.0 (curve *c*) with HCl.

excess of thioglycollic acid;⁴ (b) while the 320 m μ absorbance remains unchanged for prolonged periods (Fig. 4, curve 3; Fig. 5, curve 1), on the addition of thioglycollic acid in excess, the absorbance drops precipitously (Fig. 5, curve 1 d).

The effect of various conditions on the conversion of PNCE to PSSP was investigated, the extent of disulfide formation being estimated from the relative level of stabilization of 320 m μ absorbance. At pH 9, urea enhanced PSSP formation (Fig. 5, curves 2 and 3), especially in the presence of H₂O₂ (Fig. 5, curve 1). At pH 7, PSSP accumulation was greater than at pH 9 (Fig. 4), but enhancement by urea and H₂O₂ was only slight. H₂O₂, in the absence of urea, had no significant effect on disulfide formation at pH 7-9. Cu₂SO₄, in catalytic quantity (1×10^{-7} M), enhanced the reaction at pH 7, but was less effective than urea and H₂O₂.

The highest yield of PSSP was obtained in 0.1 to 0.01 M borate, pH 9, 8 M urea, 0.3 to 1.5 M H₂O₂, and 0.001 M versene. Versene was added to reduce possible mercaptide formation, but we have no evidence that it influenced the yield of PSSP. Because high pH favors the reaction PNCE \rightarrow PSSP, we assume the PNCE mercaptide ion is the reactive species.

The reaction of PNCE with cysteine, glutathione, mercaptoethylamine, thioglycollic acid, thioglycerol, and mercaptoethanol was followed at pH 7.4 and 9.¹ In the absence of urea and H₂O₂, the stabilization level of 320 m μ absorbance, on addition of equimolar amounts of mercaptan, was never higher than was observed with PNCE alone. In fact, under these conditions, mercaptan in excess accelerated PNCE decay, and this occurred even with equimolar amounts of mercaptoethylamine (Fig. 6 a). The possibility that the latter effect was due to reduction of PSSP or to the formation of a mercaptoethylamine- α -amide of penicilloic acid (3 i) was not investigated further.

In the presence of 8 M urea and H₂O₂, however, the 320 m μ absorbance stabilized at a higher level in a mixture of PNCE and mercaptoethylamine than with PNCE alone (Fig. 6(a)). In such a mixture, the high and stable 320 m μ absorbance dropped precipitously on addition of thioglycollic acid.

These observations support the view that a PNCE-mercaptoethylamine mixed disulfide is formed under appropriate conditions (pH 9, urea, H₂O₂). The absorption maximum of the presumptive mixed disulfide was at 320 m μ .

The 320 m μ absorbance obtained with equimolar amounts of PNCE and other mercaptans (cysteine, glutathione, thioglycollic acid, thioglycerol, and mercaptoethanol), in the presence of 8 M urea and H₂O₂ was, however, the same as with PNCE alone. Hence no evidence for the formation of PNCE mixed disulfides with these other mercaptans was obtained.

Reaction of Penicillinic Acid with p-Chloromercuribenzoic Acid.—PNCE formed a stable mercaptide with *p*-chloromercuribenzoate¹ over a wide range of pH values (4.6-9.0), even in the absence of urea. Addition of *p*-chloromercuribenzoate to PNCE results in an almost instantaneous but small drop in 320 m μ absorbance, which thereafter remains stable (Fig. 6 b). The small decrease in 320 m μ absorbance is largely ascribable to a shift of the absorption spectrum of the mercuribenzoate mercaptide which is formed (PNCMB; see Figs. 1 and 2). Since PNCMB can be obtained under conditions where PNCE degradation is minimal, this mercaptide was prepared in large quantity for use as a hapten.

⁵ Nitroprusside reactions were carried out in 0.15 μ mole NaCN in a total volume of 15 ml, and the results expressed in arbitrary units from readings in a Klett-Summerson colorimeter. The results (in parentheses) were as follows: 5 μ moles thioglycollic acid (196), 70 μ moles penicillin (0), 70 μ moles penicillin + 5 μ moles thioglycollic acid (198), 10 μ moles PNCE as an aqueous solution with stable 320 m μ absorbance (22), 10 μ moles PNCE in the latter form + 5 μ moles thioglycollic acid (390).

167 mg. PNCE (0.5 millimole) in 5 ml. 95 per cent ethanol was added to 189.5 mg. *p*-chloromercuribenzoate (0.5 millimole) in 20 ml. 0.1 M phosphate, pH 7.4. After 15 minutes at room temperature the reaction mixture was acidified to pH 4.5 with 1 N HCl and extracted with ether. Under these conditions, the ether extract contains > 95 per cent of the reaction product (PNCMB), about 95 per cent of any unreacted *p*-chloromercuribenzoate, but only about 30 per cent each of unreacted PNCE or PSSP. Drying the ether extract yielded a gummy residue which was only sparingly soluble in H₂O. Accordingly, PNCMB was extracted from the ether phase with 0.1 M KHCO₃. An absorption spectrum and dry weight determination of the final aqueous extract yielded the data of Fig. 2. The yield of PNCMB was about 75 per cent.

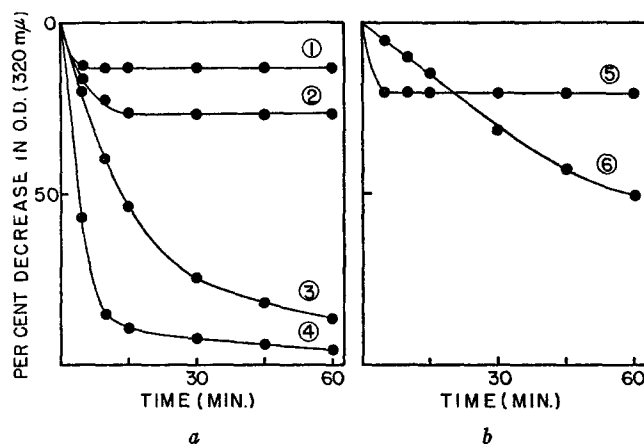


FIG. 6 *a*. Reaction of PNCE with mercaptoethylamine. A stock solution of PNCE in 95 per cent ethanol was diluted to a final concentration of 5×10^{-5} M PNCE, 1 per cent ethanol, in the following solvents: 0.01 M borate pH 9.0, 0.001 M versene, 8 M urea, 0.3 M H₂O₂ and 5×10^{-5} M mercaptoethylamine (curve 1); 0.01 M borate pH 9.0, 0.001 M versene, 8 M urea, 0.3 M H₂O₂ (curve 2); 0.01 M borate pH 9.0, 0.001 M versene (curve 3); 0.01 M borate pH 9.0, 0.001 M versene, and 5×10^{-5} M mercaptoethylamine (curve 4).

FIG. 6 *b*. Reaction of PNCE with *p*-chloromercuribenzoate. Procedure was the same as in Fig. 6 *a* except that the reaction mixtures, containing 5×10^{-5} M PNCE, 1 per cent ethanol, varied as follows:—0.01 M phosphate pH 7.4 and 5×10^{-5} M *p*-chloromercuribenzoate (curve 5); 0.01 M phosphate pH 7.4 (curve 6).

Purity of the product was indicated by the constancy of the ratio of absorbances at 234 $m\mu$ /328 $m\mu$ upon repeated partition between water and ether. A few attempts to crystallize PNCMB were unsuccessful. The compound was stable above pH 3. Extraction of the initial ether extract with 0.01 M phosphate pH 7.4–0.15 M NaCl, as desired for hapten inhibition studies, was less efficient, but became satisfactory on adding a few drops of 5 N NaOH to bring the pH of the aqueous phase to 7.4.

Reaction of Penicillenic Acid (PNCE) with Proteins and Preparation of Conjugates.—In a preliminary experiment, S³⁵-labeled PNCE of high purity was reacted with gelatin which had been artificially enriched in SH groups by reaction with *N*-acetyl homocysteine thiolactone (thiogel;¹ see reference 9). The results presented in Table II show that at pH 7.4, in the absence of urea and H₂O₂, some S³⁵ was firmly associated with the thiolated protein. Since the concentration of protein recovered was low, and a significant 320 $m\mu$ absorption peak was not detected, it could not be ascertained that the S³⁵ was actually bound as PNCE.

Subsequent experience demonstrated that in order to achieve more effective conjugation it was necessary to use large excesses of PNCE and to carry out the reaction at pH 9, in urea and H_2O_2 .

When thiolated and native proteins⁶ were reacted with PNCE under optimal conditions, the conjugates exhibited a 320 $m\mu$ absorption peak. From the absorbance at this wavelength and the biuret analysis, the number of PNCE groups per mole of protein was calculated, assuming the conjugated PNCE has a molecular extinction coefficient of 26,600.

TABLE II
*Binding of S³⁵-PNCE by Thiolated Gelatin ("Thiogel")**

Incubation time	Non-dialyzable material		Moles S ³⁵ -PNCE bound per 10 ⁶ gm. thiogel
	S ³⁵	Protein	
<i>min.</i>	<i>c.p.m.</i>	<i>mg.</i>	
10	1520	3.42	12
30	1545	3.12	13
60	1600	3.20	13
120	1710	3.41	13
720	1590	3.28	13
Control‡	40	0	—

* 3.75 mg. thiogel (32 moles SH per 10⁶ gm. protein) was incubated in 0.1 M phosphate, pH 7.4, with 4.3 μ moles S³⁵-PNCE at room temperature (total volume: 1.0 ml.), and then dialyzed at 4° for 5 days against 0.001 M phosphate, pH 7.4. Samples were dried, weighed, and counted in a gas-flow counter. The S³⁵-PNCE was prepared by Levine's method (7) from *N*-ethylpiperidine salt of S³⁵-benzylpenicillin (see footnote 8). Specific activity of S³⁵-PNCE, 11,070 c.p.m./mg.; purity, 90 per cent.

‡ Same amount of S³⁵-PNCE incubated 720 minutes without protein, and dialyzed under the same conditions.

Evidence that PNCE was covalently bound to protein through mixed disulfide linkages was as follows: (a) The PNCE/protein ratio remained constant on prolonged dialysis, treatment with an anion exchange resin (amberlite IRA-400), and on precipitation and washing the protein with organic solvents (Table III). (b) Upon addition of an excess of thioglycolic acid, the 320 $m\mu$ absorbance of the conjugate decreased rapidly. (c) The PNCE/protein ratios achieved with thiolated proteins were in close agreement with the number of SH groups on the proteins, when the conjugates were prepared with PNCE in sufficient

⁶ The various conjugated proteins have been designated with the abbreviations shown in Table III. P-S-(protein) refers to PNCE conjugates prepared with proteins artificially enriched in SH groups (thiolated) and P-(protein) to PNCE conjugates prepared by reaction with unmodified ("native") proteins.

excess (Table III). (d) The PNCE/protein ratios achieved with native proteins were in the range expected, if one assumes that disulfide groups of the native proteins undergo thiol-disulfide interchange in the presence of 8 M urea and a large excess of PNCE.

TABLE III
Reactions of Penicillenic Acid with Some Proteins*

Proteins†	Protein SH groups‡	PNCE excess (initial)	PNCE conjugated with protein	Symbol for conjugate
	<i>moles/mole protein</i>	<i>moles/mole protein</i>	<i>moles/mole protein</i>	
TG	18	930	5 (R)	P-S-TG
		1500	4 (PW)	
		1700	6 (PW)	
		5980	11 (D)	
		9270	14 (D)	
		17,040	18 (PW)	
H γ G	(35)	2025	31 (D)	P-H γ G
		2890	33 (D), 31 (PW), 35 (R)	
H γ G-SH	44	2890	36 (D), 40 (PW)	P-S-H γ G
	67	7230	72 (PW)	
B γ G	(35)	480	14 (D), 12 (PW), 12 (R)	P-B γ G
B γ G-SH	25	2555	23 (D), 23 (R)	P-S-B γ G
HSA	(35)	525	9 (D), 8 (R)	P-HSA
		2520	18 (D), 18 (R)	
HSA-SH	10	525	8 (D), 9 (R)	P-S-HSA
	28	2520	26 (D), 25 (R)	

* The reactions were all carried out at pH 9 in 8 M urea, 0.3 to 1.5 M H₂O₂ (see Experimental).

† Abbreviations are as follows: TG (thiogel: thiolated gelatin; *i.e.*, artificially enriched in SH groups (9)); H γ G (human γ -globulin); H γ G-SH (thiolated H γ G); B γ G (bovine γ -globulin); B γ G-SH (thiolated B γ G); HSA (human serum albumin); HSA-SH (thiolated HSA).

‡ SH group analysis carried out on thiolated proteins by the method of Boyer (10). —S—S— groups in the native proteins are given in parentheses as potential SH groups from data in the literature (25–27). In the case of thiogel, 10⁵ gm. is arbitrarily taken as a mole. 70,000 and 160,000 are taken as molecular weights for serum albumin and γ -globulins respectively.

|| Unreacted PNCE and its disulfide (PSSP) were separated from conjugates by several methods: prolonged dialysis (D), precipitation and washing with acetone (PW), or treatment with IRA-400 resin (R) (see experimental). The method of purification used is given in parentheses next to the value for moles PNCE/mole protein conjugate.

Some preparative details follow: Bovine serum albumin, bovine γ -globulin, human serum albumin, and human γ -globulin¹ were enriched with SH groups essentially according to the method of Benesch *et al.* (9). A fivefold molar excess (in respect to protein amino groups) of *N*-acetyl-homocysteine thiolactone (AHTL) was added to a 3 to 5 per cent protein solution. The pH was adjusted to 7.5 and AgNO₃, in an amount equivalent to AHTL, was added dropwise with stirring. The reaction was allowed to proceed for 2 to 3 hours at room temperature, with the pH maintained at 7.5 by occasional additions of 0.5 NaOH. The protein solution was then acidified to pH 2.5 with 0.5 N HCl and saturated thiourea (at pH 2.5) was added in a five- to sixfold molar excess in respect to Ag⁺ in order to obtain a clear solution. The protein was precipitated in the cold with 10 volumes acetone, washed three times with acetone, redissolved in 0.1 M acetate, pH 4-5, and insoluble gel was discarded. The protein solution was analyzed for SH groups by Boyer's method (10), and for protein by the biuret reaction, using the corresponding native protein as a standard. Even in the cold and at pH 4, these proteins were unstable, exhibiting a fast decrease in SH groups and a tendency to gel formation. They were, therefore, always freshly prepared for reaction with PNCE. Recoveries of soluble thiolated proteins were 40 to 50 per cent for the serum albumins and 60 to 70 per cent for the γ -globulins.

Thiolated and native proteins were reacted at room temperature with large excesses of PNCE (400 to 3000 molar excess in respect to protein, 5 to 40 molar excess in respect to protein SH groups). In a representative reaction, the components were added rapidly and mixed in the following order: 24 gm. urea; 350 mg. of thiolated human serum albumin (about 15 moles SH per 70,000 gm. protein) in 20 ml. of 0.01 M acetate, pH 4; 15 ml. of 0.1 M borate, pH 9, 0.01 M in respect to versene; 1.67 gm. PNCE (5 millimoles) in 7 ml. 95 per cent ethanol; 5 ml. 1 N NaOH; 2 ml. 30 per cent H₂O₂. The reaction was allowed to proceed for 1 hour, with frequent additions of 1 N NaOH to maintain the pH at 8.5-9.0. Final reaction volume: 62 ml.

The foregoing reaction conditions favor the formation of PSSP. Hence, the method used to separate this derivative and any unreacted PNCE from the conjugated protein becomes critical. The conjugated proteins precipitated when brought to pH 4.5, but repeated washing with water was insufficient to remove PSSP and PNCE, which have limited solubilities in water at low pH. On the other hand, repeated washing of the precipitates with ethanol or acetone was efficient in this respect but the proteins after such treatment were mostly insoluble. Columns prepared with anion exchange resin (amberlite IRA-400 in Cl⁻ form) retained PNCE and PSSP effectively at pH 7.4 (capacity of 1 gm. resin for PNCE and PSSP: ~4 to 5 μ moles), but the heavily conjugated proteins were extensively adsorbed by the columns, and could not be eluted easily. The most effective separation was achieved by dialyzing the reaction mixtures in the cold against frequent changes of large volumes of 0.01 M phosphate, pH 7.4, to which IRA-400 resin was added. Samples were withdrawn periodically from the dialysis bags and analyzed for protein by the biuret reaction and for PNCE by determining absorbance at 320 $m\mu$. Several days of dialysis were needed to achieve a constant 320 $m\mu$ absorbance/protein ratio, at which point the conjugates were assumed to be free of unbound PNCE or PSSP. This assumption was validated by shaking samples with IRA-400 resin (twice the amount of resin needed to remove an amount of PNCE corresponding to the total 320 $m\mu$ absorbance) or by precipitation and washing of the proteins with an organic solvent. When purified by these various procedures, the PNCE/protein ratio for a given conjugate was essentially the same (Table III). The biuret analysis (taking native proteins as standards) and dry weight of the conjugated proteins (corrected for PNCE combined) were in fair agreement.

The absorption spectrum of a typical conjugate is given in Fig. 2. As judged from 320 $m\mu$ absorbance, PNCE-protein conjugates were stable over several weeks in 0.01 M phosphate, pH 7.4-0.15 M NaCl, and for at least 2 hours in 0.25 M acetic acid (see precipitin analyses below).

Immunization of Guinea Pigs.—Guinea pigs were injected once with 1 mg. of P-S-H γ G⁶ in Freund's adjuvant (0.2 ml. into each footpad). The Freund's adjuvant mixture was prepared as previously described (11). The animals were bled after 3 weeks and a ring test was performed on individual sera with various conjugated and unconjugated proteins. Several days later, skin tests were

TABLE IV
Immunization of Guinea Pigs and Rabbits with PNCE-Protein Conjugates

Immunizing conjugate*	Animal No.	Precipitin reaction [†] with				Skin reaction [§] with				
		P-S-H γ G	P-S-TG	H γ G	TG	P-S-H γ G	P-S-TG	H γ G	TG	
Guinea pigs P-S-H γ G (1 mg. protein, 0.25 μ moles PNCE)	1	++	+	0	0	+++	+	0	\pm	
	2	++	+	0	0	+++	++	0	0	
	3	++	+	0	0	+++	+	0	0	
	4	\pm	0	0	0	+	\pm	0	\pm	
	5	++	+	0	0	+++	++	0	0	
Guinea pig controls	6	0	0	0	0	0	\pm	0	\pm	
	7	0	0	0	0	0	0	0	0	
Rabbits	P-S-B γ G (5 mg. protein, 0.68 μ moles PNCE)	1	+++	++	0	+	+++	+++	0	+
		2	+++	++	0	+	+++	+++	0	+
		3	+++	++	0	+	+++	+++	0	+
	P-B γ G (5 mg. protein, 0.25 μ moles PNCE)	4	+	+	0	++	+	+	0	+++
		5	+	+	0	++	+	+	0	+++
	Rabbit controls	6	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0

* Animals injected once with protein conjugate in Freund's adjuvant (0.2 ml. per footpad for guinea pigs; 0.4 ml. per footpad for rabbits).

[†] Qualitative precipitin reactions were obtained with 0.2 ml. serum and 40 μ g. protein in 0.5 ml. The conjugated test antigens had the following number of PNCE residues per mole protein: P-S-H γ G, 40; P-S-TG, 10; P-S-HSA, 8; P-HSA, 8. For abbreviations see legend of Table III.

[§] Intradermal injection of 25 μ g. protein in 0.1 ml. 0.01 M phosphate, pH 7.4–0.15 M NaCl. Reactions were definite at 4 to 6 hours (or sooner) and were maximal at about 24 hours. The readings recorded were made at 24 hours. +++, induration of more than 10 mm., erythema and central necrosis with hemorrhage; ++, induration of 5 to 10 mm., erythema; +, induration of 0.3 to 0.5 mm., erythema and edema; \pm , no induration, slight erythema and edema; 0, no reaction.

performed by intradermal injection of the various proteins. The results are shown in Table IV.

A quantitative precipitin analysis (see below) carried out on pooled sera with P-S-HSA⁶ as antigen, showed 300 $\mu\text{g.}$ antibody per ml. serum. Addition of 10 μmoles PNCMB to 0.5 ml. of serum inhibited precipitation completely at the equivalence point, whereas 10 μmoles of freshly dissolved benzylpenicillin inhibited only to a slight extent. The P-S-HSA antigen-antibody precipitate dissolved completely at pH 7.4 in an excess of PNCMB. Specificity of the antibody response for PNCE was evaluated in greater detail on rabbit antisera.

Immunization of Rabbits.—Fifteen rabbits were injected once with 5 mg. of P-S-B γ G⁶ and five were injected once with 5 mg. of P-B γ G,⁶ the proteins being incorporated in Freund's adjuvant (0.4 ml. per footpad). The animals were bled after 4 weeks, and 2 months later skin tests were performed on some of the animals. The results of the skin tests are summarized in Table IV. Using P-S-HSA as test antigen, qualitative precipitin tests were positive on thirteen of the animals immunized with P-S-B γ G and on four of the animals immunized with P-B γ G.

Precipitin Analysis on Rabbit Anti-PNCE Antisera.—Precipitin reactions were carried out with a constant volume of antiserum and variable amounts of PNCE-protein conjugates. Antiserum and antigen blanks, as well as all reaction mixtures were set up in duplicate. After incubation at 37° for 1 hour, the tubes were kept for 48 hours at 4°. Precipitates were washed twice with ice cold saline, air-dried, and dissolved in 0.25 M acetic acid. As a precaution, the acetic acid solutions were centrifuged before analysis, although no gross sediment was observed. The solutions were read in a Beckman DU spectrophotometer at 320 and 278 $m\mu$. The calculation of the amounts of antibody and antigen in the precipitate from the absorbances at these wavelengths is based on principles given elsewhere (11, 12). Contribution of the antigen to the 278 $m\mu$ absorbance was calculated from the conjugate's absorption spectrum in 0.25 M acetic acid. A secondary correction was made by deducting from the 320 $m\mu$ absorbance the small absorption at this wavelength due to antibody. The latter was estimated to be 3 per cent of the antibody absorbance at 278 $m\mu$ on the basis of the absorption spectrum of rabbit γ -globulin. The corrected 278 $m\mu$ absorption values were converted to milligrams of antibody, using as an extinction value for antibody $E_{1\text{ cm.}}^{1\text{ per cent}}$, 13.6 (11).

Quantitative precipitin analysis on individual sera (with P-S-HSA) revealed an antibody content ranging from 950 to 1680 $\mu\text{g./ml.}$ serum for four of the rabbits immunized with P-S-B γ G, and from 300 to 450 $\mu\text{g./ml.}$ serum for three of the rabbits immunized with P-B γ G. Representative data with a serum pool and with a γ -pseudoglobulin fraction obtained from this pool are presented in Fig. 7 and in Table V. Supernatant tests for antigen and antibody, as well as antibody/antigen ratios in the precipitates, showed that this system behaves the same as classical precipitating single antigen-antibody systems (13).

Hapten Inhibition Studies of Anti-PNCE Antisera.—Inhibition experiments were initially carried out in whole antiserum. Haptens and antiserum were mixed, and antigen (P-S-HSA) was then added in the amount required to reach the equivalence point in the uninhibited control. After 1 hour at 37° and 48 hours at 4°, precipitates were washed and analyzed as described above.

PNCMB inhibited precipitation strongly, whereas benzylpenicillin had a much smaller effect. 50 per cent inhibition was achieved at a total concentration of 7×10^{-5} M PNCMB and 3×10^{-3} benzylpenicillin. (The uninhibited control precipitate was 475 μ g. antibody and 100 μ g. P-S-HSA.)

In order to minimize non-specific binding of haptens (by serum albumin), further experiments were carried out in the same way, but with the antibody in

TABLE V
*Precipitin Reaction of Anti-PNCE Rabbit γ -Pseudoglobulin Fraction**

Antigen (P-S-HSA) added	Antigen precipitated	Antibody precipitated	Weight ratio antibody/antigen	Supernatant tests [†]	
				For antigen	For antibody
μ g.	μ g.	μ g.			
5	4.6	37.9	8.2	—	++
10	9.4	74.2	7.9	—	++
20	19.7	158	8.0	—	+
40	39.2	282	7.2	—	—
80	44.8	282	6.3	±	—
100	46.2	280	6.0	+	—
120	47.5	257	5.4	+	—
140	48.1	247	5.1	++	—
160	48.5	231	4.7	++	—
200	45.2	193	4.2	++	—
300	35.0	104	2.9	++	—

* Anti-PNCE rabbit γ -pseudoglobulin fraction was prepared from the pooled sera of ten rabbits immunized by one injection of 5 mg. P-S-B γ G (0.68 μ mole PNCE) in Freund's adjuvant. After precipitation at 1.75 M (NH₄)₂SO₄, the washed, redissolved precipitate was dialyzed in the cold for 2 days against 0.001 M phosphate, pH 7.4. The sediment which formed was discarded. The supernate is considered γ -pseudoglobulin (28). The precipitin reactions were carried out with 0.2 ml. aliquots of the γ -pseudoglobulin (about 2.8 mg. total protein) and various amounts of P-S-HSA (8 moles PNCE/70,000 gm. protein). All tubes in 0.15 M NaCl-0.01 M phosphate, pH 7.4. Total volume, 1.5 ml. Analyses for antigen and antibody in the precipitates (dissolved in 0.25 M acetic acid) were based on the 320 $m\mu$ and 278 $m\mu$ absorbances (see Experimental).

[†] Supernatant tests for antigen and antibody were carried out with 0.2 ml. aliquots of the supernatants and with 0.2 ml. γ -pseudoglobulin or 40 μ g. P-S-HSA (0.2 ml.) respectively.

the form of a γ -pseudoglobulin fraction of the antiserum. Inhibition curves with various haptens are presented in Fig. 8. PNCMB was by far the most strongly inhibiting hapten. Benzylpenicillin (PN) and the cysteine derivative of benzylpenicillin (PNCy)⁷ exhibited only weak inhibition. Penillic acid

⁷ The cysteine derivative of benzylpenicillin (PNCy) was prepared according to the Squibb procedure (3 *j*). Its crystallized benzylamine salt had a melting point (155–156°) which agreed with published figures. Crystalline benzylpenillic acid (PNI) was obtained from aqueous solutions of benzylpenicillin standing for 24 hours at pH 2.5 at room temperature. Its melting point (188–189°) corresponded to data in the literature (3 *e*).

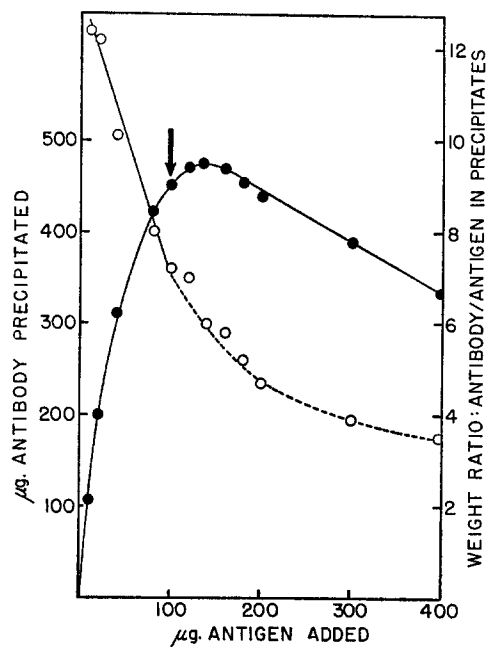


FIG. 7. Specific precipitation of anti-PNCE rabbit antiserum. Each reaction tube contained 0.5 ml. of a pooled serum from rabbits immunized with P-S-B γ G (see Experimental). The test antigen was P-S-HSA (8 moles PNCE/70,000 gm. protein). Total volume per tube: 1.5 ml. (●) represents micrograms of antibody precipitated and (○) represents weight ratios antibody/P-S-HSA in the corresponding precipitates. Equivalence point (↓) obtained from supernatant tests (see Table V for procedure).

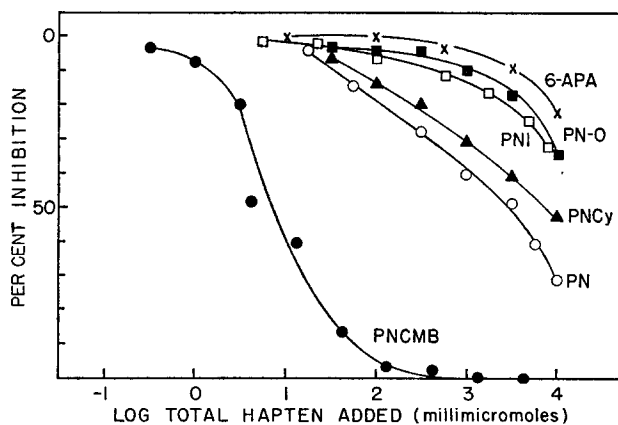


FIG. 8. Inhibition of precipitation by haptens. Precipitation reactions were set up, in duplicate, at the equivalence point with 0.4 ml. rabbit γ -pseudoglobulin fraction (580 μ g. antibody) and 0.5 ml. P-S-HSA conjugate (100 μ g.; 11.4 m μ eq. PNCE substituents) in the presence of various amounts of hapten. Final volume: 2 ml. Fifty per cent inhibition required 7 m μ moles PNCMB, or 2600 m μ moles PN, or 7800 m μ moles PNCy. For the structure of the haptens, and for abbreviations, see Fig. 1.

(PNI),⁷ allylthiomethylpenicillin (PN-O),¹ and 6-aminopenicillanic acid¹ (6-APA) had very slight inhibitory effects and only at high concentrations. None of these substances, at the maximal concentration used (5×10^{-8} M), inhibited the precipitation by egg albumin of an anti-egg albumin rabbit γ -globulin fraction.

DISCUSSION

Guinea pigs and rabbits immunized with PNCE-protein conjugates develop substantial amounts of serum antibodies and pronounced allergic skin reactivity, presumably of the Arthus type. The regularity of these responses and their intensity indicate that the PNCE group is comparable in its antigenic potency to other effective low molecular weight determinants; *e.g.*, the 2,4-dinitrophenyl group (12). It is apparent from quantitative inhibition data (Fig. 8) that the antibodies precipitated by PNCE-protein conjugates have a high affinity for the oxazolone structure (PNCMB).⁸ Levine has suggested that PNCE might conjugate with protein through acylation of free NH_2 groups, in which case the determinant would be essentially an α -amide of penicilloic acid (14). Since specific precipitation of the present system was completely inhibited by PNCMB, either the substitution suggested by Levine does not occur under the conditions used herein to prepare conjugates, or the penicilloate α -amide substituents do not induce antibody formation.

The inhibition exerted by both benzylpenicillin (PN) and by allylthiomethylpenicillin (PNO) cannot be interpreted readily, as some conversion of these substances to the corresponding penicillenic acids takes place during the incubation period. In fact, the allylthiomethylpenicillin (PNO) preparation used in these experiments contained a 320 $m\mu$ absorbance band to begin with, indicating some contamination with a penicillenic acid. However, at the beginning of incubation benzylpenicillin had no 320 $m\mu$ absorbance and yet its inhibition of precipitation was apparent after a few minutes, when only negligible amounts of PNCE could have been formed. PNCy, which does not rearrange to PNCE, was also slightly inhibitory. Since the derivatives with least inhibiting activity are devoid of the benzyl side chain (PNO, 6-APA), it is possible that the antibodies induced by benzyl-PNCE conjugates have some slight specificity for the benzyl group. In view of the virtual absence of inhibition by 6-APA, it is clear that anti-PNCE antibodies have no affinity for the β -lactam ring and the thiazolidine ring of penicillin.⁹

⁸ In a preliminary qualitative experiment, the oxazolone moiety of PNCE (2-benzyl-4-hydroxymethylene-(5)-oxazolone, Na salt) inhibited precipitation of anti-PNCE serum by P-S-HSA to an appreciable extent. Quantitative measurements of the degree of inhibition will be required for evaluation of the relative contribution of the oxazolone and penicillamine moieties of PNCMB to the specific interaction with anti-PNCE antibody. Crystalline benzyl-oxazolone was prepared from PNCE by alkaline degradation (3 *k*).

⁹ Actually, except for PNCMB and PN, the inhibition of precipitation by all the substances

It is difficult to evaluate directly the determinant involved in the allergic skin reactions. Nevertheless, from the responses to various proteins and protein conjugates (Table IV), it is quite clear that the PNCE group is a major determinant in some of these reactions.

PNCE conjugates prepared with native proteins were less effective as immunizing agents than conjugates prepared with thiolated proteins. It is tempting to suppose that the thiolated conjugates, which leave the PNCE substituents more extended from the protein main chain, are more effective inducers because the PNCE determinant is sterically more available. However, the difference in the number of substituent groups on the two conjugates used for immunization was so great (8 per mole protein for P-B γ G, 22 per mole protein for P-S-B γ G) that the basis for their unequal efficiency is not yet clear. As might be expected, the conjugates which induced the smaller anti-PNCE response induced a greater response to the protein moiety (B γ G).

From the data concerning PNCE formation from penicillin, it is clear that under conditions of conventional clinical usage, individuals injected with penicillin are exposed to PNCE. For example, benzylpenicillin solutions at a concentration of 300,000 units/ml. yield significant amounts of PNCE after a short time at room temperature (in saline, pH 4.7, 1.1 mg./ml./hour; in buffered saline, pH 7.4, 30 μ g./ml./hour).¹⁰ Furthermore, the capacity of the PNCE sulfhydryl to enter into mixed disulfide linkages with protein SH groups makes it highly probable that PNCE conjugation occurs *in vivo*.¹¹ Finally, the potency

tested (PNCy, PN-O, PNI, and 6-APA) is best regarded, provisionally, as total absence of inhibition. Trace contamination of the latter ineffectual substances by undetectable amounts of penicillenic acids (< 0.1 per cent by weight) could account for the slight inhibition exerted at very high concentrations.

¹⁰ These figures are calculated from the initial rates of conversion of benzylpenicillin to PNCE and correspond to a decrease in antibiotic activity (respectively 0.5 per cent and 0.015 per cent per hour) which is insignificant in therapy, but quite meaningful if the derivative formed is a powerful sensitizer. Although the conversion of penicillin to PNCE *in vivo* has not been ascertained, it should be noted that the reaction is accelerated at 37°.

¹¹ The following indirect evidence supports the view that PNCE is actually able to conjugate *in vivo*: (a) Levine has induced contact sensitivity to PNCE in guinea pigs by intradermal injection of PNCE (14). (b) PNCE is apparently bound by bacterial extracts (17). (c) In 0.15 M NaCl, at pH 7.4, red blood cells are hemolyzed when incubated with PNCE (0.8 to 1 mg./ml.) whereas they withstand much greater concentrations of benzylpenicillin (20 to 30 mg./ml.).

In addition, preliminary experiments performed with S³⁵-labeled penicillin suggest that PNCE derived from penicillin can conjugate with serum proteins. Radioactive S³⁵-benzylpenicillin was prepared by fermentation in a synthetic medium (19-21) of Q-176 *Penicillium chrysogenum* (obtained by courtesy of Dr. C. W. Hesseltine, Agricultural Research Service, Peoria, Illinois). The S³⁵-benzylpenicillin was isolated as *N*-ethylpiperidine salt (22) of constant specific activity. Incubation of the S³⁵-penicillin with proteins at various pHs and temperatures was followed by precipitation of the protein with (NH₄)₂SO₄, washing, and prolonged dialysis (sufficient to remove completely S³⁵-penicillin in the absence of protein). A substantial

of PNCE-protein conjugates as immunizing agent is such that anti-PNCE antibodies can be expected with some confidence in at least some individuals exposed to penicillin.

On the basis of this argument, it becomes of interest to consider the specificity of the presumptive anti-penicillin antibodies detected by hemagglutination techniques in human and rabbit sera. Red blood cells, preincubated for 1 hour at 37° with penicillin, are agglutinated by some sera of individuals allergic to penicillin (16), as well as by serum of rabbits injected with large amounts of penicillin in Freund's adjuvant (15). The possibility that, under such conditions, the cells are actually tagged with PNCE, cannot be excluded. Similarly, the apparent inhibition of the hemagglutination by penicillin could be due to PNCE and its corresponding disulfide, formed during the assay. Even if this were not the case, the apparent reactivity of anti-PNCE antibodies with penicillin (while small) could account for the hemagglutination reaction itself, and for the inhibition of the reaction by penicillin (15, 16). In any event, the present experiments make it necessary to reevaluate the significance of the hemagglutination by some sera of penicillin-treated red blood cells.

The foregoing arguments do not mean that the formation of considerable amounts of anti-PNCE antibodies is an obligatory consequence of penicillin injection. The rates of penicillin → PNCE rearrangement, the dilution of PNCE, and rate of PNCE conjugation *in vivo*, etc., are all factors which make the situation in human subjects given penicillin very different from that in animals injected with preformed highly substituted conjugates in Freund's adjuvant.

Although the possible role of PNCE in the induction of penicillin hypersensitivity in man has been emphasized in the present discussion, it must be stressed that no direct evidence bearing on this possibility has yet been obtained. If it should turn out that PNCE is a significant antigenic determinant in penicillin hypersensitivity, a means for screening various penicillins for their allergenic potential may be visualized by determining the rates of their conversion to the corresponding penicillenic acids, and the reactivities of these derivatives. The extraordinary variety of known breakdown products of penicillin must, however,

amount of S³⁵ activity was associated with human serum albumin (23, 24), but the binding was independent of temperature and pH (4.6–9.0) and was presumably reversible (24). No S³⁵-penicillin was bound by human or bovine γ -globulin at pH 7.4. However, upon incubation at pH 4.6 (which favors the penicillin → PNCE conversion), some binding of S³⁵ occurred and was more marked at room temperature than at 4°. The assumption that the S³⁵ was bound as PNCE was substantiated by the observation of a similar degree of binding with S³⁵-PNCE (formed and isolated from S³⁵-penicillin) at pH 7.4 as well as at pH 4.6. The amounts of penicillin and PNCE used for incubation were small (30 to 55-fold mole excess in respect to protein) and only 1 mole S³⁵ per mole protein was bound. Native γ -globulins apparently have only one free SH group per mole (25, 26).

be borne in mind. Moreover, the possibilities for numerous catalyzed reactions *in vivo* are obviously very great. Hence, even if PNCE is a determinant, the possibility will certainly remain that other derivatives, structurally unrelated to PNCE, may be involved in penicillin hypersensitivity.

SUMMARY

Under mild conditions, benzylpenicillin in aqueous solution rearranges to benzylpenicillenic acid, which reacts, under suitable conditions, to form stable disulfides and mercaptides. Through reaction with native proteins or especially with proteins that are enriched in SH groups ("thiolated"), penicillenic acid forms, *via* mixed disulfide linkages, stable highly substituted conjugates.

These conjugates are potent antigens, inducing the formation of substantial amounts of antibodies specific for the penicillenic acid structure. The implications of these findings for penicillin hypersensitivity in man are discussed.

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