The specificity of hemagglutination reactions in human and experimental penicillin hypersensitivity

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Despite considerable evidence that the penicilloyl† group is an important antigenic determinant in penicillin allergy,[1, 2, 3] there remains the problem of determining how frequently other penicillin derivatives are involved. In an earlier study, a thiolated human serum albumin containing penicillenate and other penicillin derivatives, but not penicilloyl, elicited immediate cutaneous responses in a substantial number of human subjects allergic to penicillin.[1] Because unambiguous hapten inhibition of these responses was sometimes not observed, their specificity remained open to question in certain instances. Moreover, in a variable percentage of subjects with a history of penicillin hypersensitivity, cutaneous responsiveness to penicilloyl and penicillenate antigens has not been observed.[4, 5] Occasionally, cutaneous reactions have been elicited

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†Unless otherwise specified, reference is made to benzylpenicillin and its derivatives. Structural formulas of the most relevant derivatives are given in Fig. 1. Structures are referred to by Roman numeral as well as by name and are numbered in order as they appear in the *Materials and methods* section. Abbreviations of certain of the derivatives are also given in this section. A given Roman numeral is used to refer to the salt of a carboxylic acid as well as to the free acid. Additional structural formulas are shown in Fig. 3 and are numbered consecutively as they appear in the *Discussion* section, beginning with XXVI.

by penicillin in such patients, suggesting that penicillin derivatives other than penicilloyl and penicillenate may be responsible for hypersensitivity.[4]

At present, hemagglutination is the only technique that allows the use of a variety of penicillin derivatives for evaluation of the antibody specificity of a large number of individual human sera in relatively small quantities. Although hemagglutination has been utilized previously in the study of human penicillin allergy,[6-12] a detailed evaluation of specific hemagglutination inhibition by those penicillin derivatives capable of existing in covalent linkage to protein has not been published. In our study, a modification in the technique of hemagglutination made it possible to carry out extensive observations of hapten inhibition with a relatively high proportion of sera.

Sera from 114 subjects with penicillin allergy were evaluated for their reactivity and specificity in hemagglutination by means of hapten inhibition, and the results were compared to those obtained with rabbit antisera to penicillin and to penicilloyl- and penicillenate-substituted proteins. The results show that the penicilloyl group is an important antigenic determinant in hemagglutination reactions mediated by sera from subjects with penicillin hypersensitivity and further indicate that there are antibodies of at least three additional specificities in the sera of subjects sensitive to penicillin.

MATERIALS AND METHODS

Penicillin and penicillin derivatives

Benzylpenicillin potassium salt (I, penicillin) was provided by Dr. D. G. Iezzoni (Chas. Pfizer & Co., Inc., Brooklyn, New York). 6-Aminopenicillanic acid (II, 6-APA) and phenethicillin were supplied by Dr. J. E. Doyle (Bristol Laboratories, Syracuse, New York). Penicillin O (allylthiomethyl) was a gift from Dr. F. T. Johnson (The Upjohn Company, Kalamazoo, Michigan).

Benzylpenicillenic acid (III, PNCE) was prepared as described previously.[13-15]

D-α-Benzylpenicilloyl-aminocaproate (IVa, D-α-benzyl-PNO-AC) was prepared by the reaction of benzylpenicillin with aminocaproate at pH 10.7 and was crystallized as the dibenzylammonium salt—once from butanol-ether and two times from ethanol-ether, m.p.* 110 to 111° C., $[\alpha]_D^{25}$ + 80° (water, c = 1.0).[16, 17]

Benzylpenicilloyl-aminocaproate also was prepared by reaction of penicillenic acid with aminocaproate (IVb).[18] The molar extinction coefficient with $HgCl_2$ at 282 mμ was 21,500, and, based on 280 mμ absorbance, the penamaldate content was 4 per cent, $[\alpha]_D^{25}$ + 50° (water, c = 0.5). A mixture of diastereoisomers of benzylpenicilloyl-aminocaproate (IVc, muta-benzyl-PNO-AC) was prepared by refluxing IVa at pH 5.0, $[\alpha]_D^{25}$ + 25° (water, c = 0.5).[16] The penamaldoyl content was 1 per cent, based on 280 mμ absorbance.

D-α-Penicilloyl-α-benzylamide (V, benzyl-PNO-benzylamide) dibenzylammonium salt was crystallized twice from ethanol-ether, m.p. 134 to 135° C.[19a]

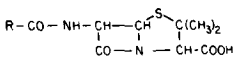
Allylthiomethylpenicilloyl-aminocaproate (VI, allylthiomethyl-PNO-AC) was

*All melting points were determined with a Fisher-Johns hot stage and are uncorrected. Optical rotations were determined with a Kern full-circle polarimeter.

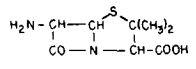
crystallized as the dibenzylammonium salt from ethanol-ether, m.p. 97 to 98° C., $[\alpha]_D^{25}$ + 102°.[16]

D-α-Benzylpenicilloate (VII) was crystallized as the monosodium salt from acetone-water, m.p. 156 to 157° C.[19b]

Benzylpenillic acid (VIII) was prepared by rearrangement of benzylpenicillin in acid solution and crystallized from water, m.p. 186 to 187° C.[19c]



I



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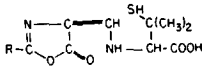

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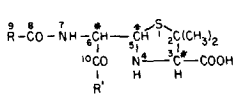
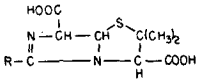


IV, V, VI, VII



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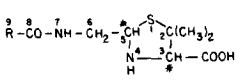
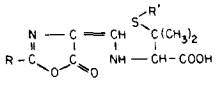
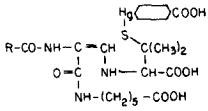


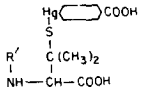
IX



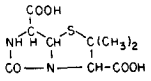
X, XI, XII



XIII



XIV, XV, XVI



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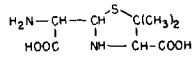

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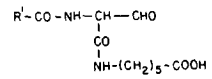

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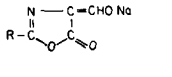
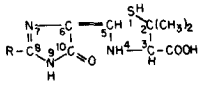
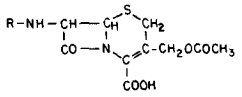


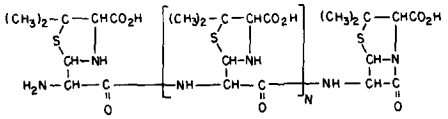
XXI



XXII



XXIV, XXV



XXVI

Fig. 1

Structural formulas of penicillin and penicillin derivatives. (For abbreviations see *Materials and methods*. See also the first footnote in the text.)

I, Benzylpenicillin, R is $C_6H_5CH_2$; *II*, 6-APA; *III*, Penicillenic acid; *IV*, Penicilloyl-aminocaproate, R′ is $NH(CH_2)_5COOH$; *V*, Penicilloyl-benzylamide, R′ is $NHCH_2C_6H_5$; *VI*, Allylthiomethyl-penicilloyl-aminocaproate, R is $CH_2{=}CH{-}CH_2SCH_2{-}$, R′ is $NH(CH_2)_5COOH$; *VII*, Penicilloic acid, R′ is OH; *VIII*, Penillic acid; *IX*, Penilloic acid; *X*, PNCE-MB, R′ is HgC_6H_4COOH; *XI*, PNCE-NES, R′ is $C_6H_5NO_2$[18]; *XII*, PNCE-PM, R′ is HgC_6H_5; *XIII*, Penamaldate-AC; *XIV*, Penillamine-mercuribenzoate, R′ is remainder of penillic molecule minus COOH; *XV*, Penicillamine-MB, R′ is H; *XVI*, Penilloic-MB, R′ is remainder of penilloic molecule (residues 5 to 9); *XVII*, 8-Hydroxypenillic acid; *XVIII*, Penicic acid; *XIX*, Penaldate-AC; *XXI*, 2-Benzyl-4-hydroxymethylene-5-oxazolone; *XXII*, Imidazolone derivative of penicilloyl-α-amide; *XXIV*, 7-ACA, R is H; *XXV*, Cephalosporin C, R is α-aminoadipic acid; *XXVI*, Poly-6-APA, N averages about 4 or 5.

D-Benzylpenilloic acid (IXa) was prepared by reflux of an acid aqueous solution of benzylpenicillin and crystallized from water, m.p. 95 to 97° C., $[\alpha]_D^{25}$ + 55° (c = 0.46 in ethanol).[19d] D-β-Benzylpenilloic acid (IXb), m.p. 164 to 166° C., was obtained from the Aldrich Chemical Co., Milwaukee, Wisconsin.[19e]

S-(*p*-Mercuribenzoate)-benzylpenicillenate (X, PNCE-MB) was prepared as described previously.[15, 18] It had a molar extinction coefficient of 22,500 at 327 mμ and did not contain detectable amounts of penicilloyl. Based on the absorbance at 280 mμ [as contrasted with the parent PNCE (III)], the quantity of penamaldate present might have been as much as 6 moles per cent. The calculated values for per cent composition by analysis* for $C_{23}H_{22}N_2O_6S_1Hg_1$ are C, 42.13; H, 3.36; and N, 4.27. The actual values found were C 41.93; H, 3.88; and N, 4.09.

S-(*N*-Ethylsuccinimidyl)-benzylpenicillenate (XI, PNCE-NES),[18] PNCE (III), 3.2 mM, and *N*-ethylmaleimide, 3.2 mM,† were dissolved in ethanol, and 1.8 volumes of 0.2M K_2HPO_4 were added slowly with mixing. After 30 minutes, the product was purified to a constant molar extinction coefficient (15,200 at 322 mμ in ethanol) by repeated acid precipitation and dissolution in 0.2M dipotassium phosphate. Based on the absorbance at 280 mμ, the penamaldate contamination might have been as much as 10 moles per cent. Calculated values for analysis of $C_{22}H_{25}N_3O_6S_1$ are C, 57.26; H, 5.43; and N, 9.11. The values found were C, 56.76; H, 5.56; and N, 8.76.

S-(Phenylmercuri)-benzylpenicillenate (XII, PNCE-PM) was prepared by the addition of freshly prepared benzylpenicillenic acid to a solution containing an excess of phenylmercuric hydroxide,[20] benzene, and glacial acetic acid at 4° C. After a few minutes the penicillenate mercaptide was precipitated from solution by the addition of petroleum ether and then washed with petroleum ether. The molar extinction coefficient at 328 mμ was 22,400 and the absorbance at 280 mμ was 10 per cent of that at 328 mμ, only 1 per cent higher than the 280/322 of the parent benzylpenicillenic acid (PNCE, III).

S-(*p*-Mercuribenzoate)-benzylpenamaldoyl-aminocaproate (XIII, penamaldate-AC) was prepared by treatment of D-α-benzylpenicilloyl-aminocaproate disodium salt with the calculated amount of *p*-hydroxymercuribenzoate shortly before use. In an effort to exclude the presence of free *p*-hydroxymercuribenzoate from the hapten stock, a 3 per cent excess of penicilloyl, as defined by titration with mercuribenzoate, was used.[16] On the basis of ultraviolet absorption, the product was stable for at least 6 hours at room temperature. In a similar manner, mercuribenzoate derivatives of benzylpenillic acid (XIV, penillamine-MB), penicillamine (XV, penicillamine-MB), and benzylpenilloic acid (XVI, penilloic-MB) were prepared. 8-Hydroxypenillic acid (XVII) was prepared by reaction of 6-APA (II) with CO_2 in neutral aqueous solution. The product was purified by chromatography on Dowex-50 (H^+ form) and lyophilized, m.p. 136 to 140° C.[21] Penicic acid disodium salt (XVIII) was prepared by alkaline

*Analyses were performed by Micro-Tech Laboratories, Skokie, Ill.
†Sigma Chemical Co., St. Louis, Mo.

Table I. *Hemagglutination titers of human sera. Correlation with previous history of allergy and skin test data*

*Skin test results**	*Hemagglutination titer†*							
	< 4	*4*	*8*	*16*	*32*	*64*	*128*	*256 or >*
Previous allergic reaction to penicillin								
Positive	1	4	2	5	7	4	1	5
Negative	0	2	2	2	2	0	0	0
Not tested	4	6	8	6	8	3	4	9
No previous allergic reaction to penicillin								
Positive	2	2	6	6	5	4	2	2
Negative	16	8	0	0	2	1	0	1
Not tested	17	12	9	1	0	1	0	0

Cells were prepared by incubation with penicillin at pH 8.6 as described in the text.

*Determined by intradermal injection with penicilloyl-polylysine, usually at a level of 6 to 12 × 10^{-5} M as described previously.[1]

†Given as the reciprocal of the greatest dilution giving a positive response. Dilutions of less than 1:4 were not evaluated.

hydrolysis of 6-APA in a manner similar to that used for the preparation of benzylpenicilloic acid from benzylpenicillin.[22]

The benzylpenaldate derivative of benzylpenicilloyl-aminocaproate (XIX, penaldate-AC) was prepared from mercuric chloride and penicilloyl-aminocaproate (IVa) as described previously.[18]

Benzylisopenillic acid (XX) was prepared by reflux of benzylpenillic acid for 20 hours in methanol followed by crystallization from methanol, m.p. 176 to 178° C.[19f]

2-Benzyl-4-hydroxymethylene-5-oxazolone (XXI) was prepared by treatment of benzylpenicillenic acid with sodium hydroxide[19g] followed by recrystallization from methanol–ethyl acetate, m.p. 235 to 239° C.

A crude imidazolone (XXII) was prepared from benzylpenicilloate-α-amide[19h] by heating at 80° C. in the presence of dilute NaOH for 30 minutes followed by acid precipitation and washing with water. The product had an absorption maximum at 310 mμ.

Penicillaminic acid (XXIII) was obtained from Calbiochem, Los Angeles, California. 7-Aminocephalosporanic acid (7-ACA, XXIV) was provided by Eli Lilly and Company, Indianapolis, Indiana, and by Glaxo Research, Ltd., Stoke Poges, Bucks, England. The latter also provided cephalosporin C (XXV). A polymer of 6-APA (poly-6-APA, XXVI) was prepared as described by Grant and colleagues.[23]

Antisera

Human sera. Control human sera were obtained from naval reservists (healthy men of various ages), from patients in an allergy clinic for adults, and from hospitalized patients of the Medical Service at Barnes Hospital.

Sera from patients with penicillin allergy were obtained at the St. Louis City Health Clinic through the courtesy of Dr. Shapiro and Dr. Summar; from Great Lakes Naval Station through the courtesy of Dr. Rytel, Dr. Ar-

lander, and associates; and from patients studied at Barnes Hospital. Among the total group of 114 sera in this category, eighty-five had been obtained from persons with a history of penicillin allergy. In about 80 per cent of instances, the penicillin reaction had been observed by a physician. In about 44 per cent of this group of eighty-five subjects, cutaneous reactivity to penicilloyl-polylysine also had been evaluated (Table I). The remaining 29 patients were considered to be allergic to penicillin on the basis of intradermal reactions to penicilloyl-polylysine; the justification for including them in the penicillin allergy category is the greatly increased risk of reaction upon subsequent administration of penicillin for these patients.[1]

Animal sera. The rabbit antipenicillenate antiserum (rabbit anti-PNCE serum) was that described previously, containing 1.7 mg. antibody per milliliter.[1]*

A pool of rabbit antipenicilloyl antiserum (rabbit anti-PNO serum) was obtained by cardiac puncture 1 month after injections distributed among the footpads of 4.8 mg. of penicilloyl–bovine gamma globulin in 1.6 ml. complete Freund's adjuvant. This antigen had been prepared by reaction of penicillenic acid with bovine gamma globulin at pH 8 to 8.5 followed by treatment with 2-mercaptoethanol and dialysis.[18] The antigen was calculated to contain 72 penicilloyl groups per molecule (based on titration with $HgCl_2$ and dry weight) and did not have an absorption shoulder in the 320 mμ region. Sera obtained at 1 month that contained substantial amounts of antibody were pooled. On titration with penicilloyl–bovine serum albumin (30 groups per molecule), there was 0.7 mg. antibody per milliliter. Precipitation of the antiserum by penicilloyl–human serum albumin was completely inhibited by D-α-penicilloyl-aminocaproate at a concentration of 1×10^{-3} M.

Individual rabbit antisera were also obtained from animals which had received 100 mg. benzylpenicillin in complete Freund's adjuvant 3 weeks previously (0.3 ml. per footpad and 0.2 ml. in four dorsal sites) and 50 mg. in 0.4 ml. complete Freund's adjuvant in four dorsal sites 1 week previously.

Rabbit antisera to human gamma globulin. Human gamma globulin (Lederle Laboratories, Pearl River, New York) was purified by DEAE-chromatography and sodium sulfate precipitation. Rabbits received injections distributed among the footpads of 4.0 mg. of purified human gamma globulin in complete Freund's adjuvant (total volume 1.6 ml.). A serum pool obtained by cardiac puncture 21 to 30 days after injection was estimated to contain 1.24 mg. antibody per milliliter by quantitative precipitin analysis. On immunoelectrophoresis,[24] single bands in the gamma-1 and gamma-2 regions were noted with human serum.

Gamma globulin fractionation

Gamma globulin fractions of human or rabbit antisera were prepared as described previously.[1] Recovery of hemagglutinating activity was generally

*Rabbit anti-PNCE serum is rabbit antiserum specific for penicillenate–thiolated bovine gamma globulin. Rabbit anti-PNO serum is rabbit antiserum specific for penicilloyl–bovine gamma globulin.

quantitative with the use of a final ammonium sulfate concentration of 40 or 50 per cent of saturation for precipitation.

Hemagglutination reactions

Preparation of human cells with penicillin. A single donor with group O, Rh-negative blood was the source of cells. Freshly drawn blood was mixed with an equal volume of Alsever's solution and the cells were washed three or four times with 0.145M NaCl and 0.01M phosphate, pH 7.4 (phosphate-saline). One volume of a 16 per cent suspension of cells in phosphate-saline was then mixed with 2.5 volumes of barbital buffer 0.14M, pH 8.6. To 3.5 volumes of this mixture was added 0.5 volume of a 400 mg. per milliliter solution of benzylpenicillin in phosphate-saline. For the control red cells, 0.5 volume of phosphate-saline was substituted for the solution of penicillin in phosphate-saline. The penicillin solution was freshly prepared, and the addition was made with constant mixing. In some experiments, 0.15M phosphate (pH 6.0 or 7.4), or 0.145M saline and 0.01M phosphate (pH 7.1 or 7.4), was substituted for the equivalent volume of barbital buffer. The whole blood of the donor mixed with an equal volume of Alsever's solution and solid penicillin was also used in some experiments.[6]

Cells were incubated with and without penicillin in these solvents for 1 hour at 37° C. and for 16 to 40 hours at 4° C. They were then washed with phosphate-saline, the amount of washing varying with the extent of hemolysis. At least three or four washes were made after the first wash that rendered the supernatant solution grossly free of hemoglobin. Cells were then stored at 4° C. in phosphate-saline and could be used for as long as 1 week thereafter. Cells were always washed at least several times more on the day of use.

Preparation of rabbit cells with penicillin. Rabbit cells were similarly prepared, with the exception that 0.5 volume of a 20 mg. per milliliter solution of benzylpenicillin was used rather than the equivalent volume of a 50 mg. per milliliter solution as previously described.[3] Use of the lower concentration of benzylpenicillin was necessary to avoid extensive hemolysis. Rabbit cells were obtained from a single donor animal.

Penicillenate-treated cells. One volume of a 16 per cent suspension of red cells was mixed with 3.8 volumes 0.14M barbital, pH 8.6, at room temperature. Benzylpenicillenic acid (0.2 volume) in 95 per cent ethanol (20 mg. per milliliter) was added drop by drop with thorough mixing. The final mixture was incubated at 37° C. for 1 hour and at 4° C. for 2 to 16 hours and washed in the manner described for penicillin-treated cells. The use of penicillenate in larger amounts resulted in excessive cell lysis.

Mercuribenzoate-treated cells. To 1 volume of a 10 per cent suspension of cells* in 0.14M NaCl and 0.01M phosphate, pH 7.4, were added 6.2 volumes of 0.145M NaCl and 0.01M phosphate, pH 8.2, and 0.8 volume of *p*-hydroxymercuribenzoate in 0.14M NaCl and 0.01M phosphate, pH 8.2; the *p*-hydroxymercuribenzoate was usually at a concentration of 5×10^{-4} M. After 30 minutes at room temperature, the cells were washed three times with phosphate-saline.

*The cells had been previously incubated with penicillin or penicillenic acid and washed as described in preceding sections.

Technique of hemagglutination. Serial dilutions of serum or gamma globulin fractions were made in phosphate-saline. In many instances, especially with sera of high titer, direct dilutions were made in order to exclude the possibility of pipette carry-over. Where comparisons of the relative inhibitory effect of various haptens were being made, dilutions of gamma globulin fractions were made in a single row of tubes and aliquots of each dilution were transferred to additional rows of tubes and mixed with the appropriate hapten solutions.

In detailed hapten-inhibition studies the highest dilution of gamma globulin which would still give marked hemagglutination was usually chosen. The dilution of gamma globulin clearly had some effect on the concentration of a given hapten required for marked to complete inhibition; this was apparent when inhibition over a broad range of gamma globulin and hapten concentrations was studied. In the inhibition studies an attempt was made to evaluate both partial and complete inhibition. With most sera, a relatively sharp progression from marked agglutination to little or no agglutination over a range of one to two dilutions of hapten (a change in hapten concentration of three- to sixfold) was noted, permitting ready comparison of the relative inhibitory capacity of various haptens. Less commonly, partial inhibition extended over a broad range in concentration of a given hapten, rendering a comparison of various haptens more difficult.

Hapten concentrations were usually varied from 1×10^{-5} (or lower, where marked inhibition had been previously noted) to 1×10^{-3} with one and one-half to threefold decreases in hapten concentration from one dilution to the next. Hapten solutions were diluted in phosphate-saline. Solutions of various penicillin and of 6-aminopenicillanic acid were freshly prepared. Dibenzylammonium salts of penicilloyl derivatives were passed through Dowex-50 (Na^+ form) at neutral pH to remove benzylammonium ions, titrated with $HgCl_2$ or *p*-hydroxymercuribenzoate (Sigma Chemical Co., St. Louis, Mo.), and the optical rotation measured. Where comparisons of various benzylpenicilloyl-aminocaproate preparations were being made (see above), the concentration and optical rotations of stock solutions were redetermined on the day of titration and fresh dilutions made. Stock solutions could be stored at –20° C. for several weeks with little or no change in optical rotation or penicilloyl content.

Gamma globulin fractions were incubated with hapten for at least 15 minutes before adding cells. To 0.2 ml. of the serial dilution of serum in saline was added 0.2 ml. 0.5 per cent freshly washed red cells. In hapten-inhibition studies the final volume of diluted gamma globulin plus hapten was 0.3 ml., to which 0.1 ml. of a 1 per cent suspension of cells was added. The tubes were thoroughly shaken and read by settling pattern by at least two observers after 1 to 2 hours at room temperature. Incubation at 37° C. for 1 hour or at 4° C. overnight had no appreciable effect on observed titers, except that hapten inhibition was sometimes less striking following prolonged incubation.

Hemagglutination reactions with rabbit antisera were carried out as previously described. In some instances rabbit antisera absorbed with untreated cells of the human O-negative donor were reacted with sensitized human cells. Rabbit antisera also were absorbed with cells of the donor rabbit before use.

Preimmunization rabbit sera absorbed with cells of the donor rabbit did not agglutinate penicillin-treated cells.

Reproducibility of hemagglutination. Fresh preparations of cells were routinely checked with standard sera from previous titrations. The variation of titer with individual batches of cells was either one dilution (twofold) above or one dilution below the usual titer. Retitration with repeatedly washed cells over a 1 week period failed to indicate any significant change in titer.

All sera were evaluated with both control cells and penicillin-treated cells. Sera with positive hemagglutination reactions were also routinely evaluated for hapten inhibition of the reaction. The specificity of the hapten-inhibition results was indicated by the failure of haptens in relatively high concentrations to affect unrelated hemagglutination systems (Table II). Observations were validated by duplicate determinations on a single day or by repetition of the experiment under the same conditions on another day. All observations of special interest (e.g., unusual inhibition patterns) were documented a number of times. The absolute concentrations of hapten required for inhibition on different days were consistent, provided that the same dilutions of gamma globulin were used.

Radioactive antigen

Radioactive bovine serum albumin was iodinated with I^{131} by the method of Staub and colleagues.[25] After treatment with IRA-400 and dialysis, the prod-

Table II. *Relative effectiveness of penicillin derivatives as inhibitors of hemagglutination with gamma globulin fractions from subjects allergic to penicillin**

Haptens used for inhibition†	*Number of sera evaluated*	*Hapten concentration required for inhibition*‡			
		1 to 10	*10 to 100*	*100 to 500*	*> 500*
Benzylpenicillin (I)	36	0	3	7	26
6-APA§ (II)	30	0	1	5	24
D-α-Benzyl-PNO-AC (IVa)‖	36‖	5	19	10	2
Muta-benzyl-PNO-AC (IVc)	26	0	12	12	2
PNCE-MB (X)	36	0	5	12	19
Penillate (VIII)	18	0	0	3	15
Benzylpenilloate (IXa)	24	1	3	8	12
Penamaldate-AC (XIII)	21	0	8	13	0
Penicillamine-MB and penicillaminic acid (XV, XXIII)	36	0	0	1	35

*In a few instances the inhibition experiments were done with unfractionated sera.

†The specificity of hapten inhibition was validated by the absence of inhibition in three unrelated hemagglutination systems (A, B, and N) at hapten concentrations as high as 2×10^{-2} M.

‡Hapten concentrations are expressed as the lowest concentration in millimicromoles per milliliter which gave marked to complete inhibition of agglutination.

§For abbreviations see *Materials and methods.*

‖All sera with titers of 1:16 or higher were evaluated for inhibition by this derivative, but in forty-nine instances inhibition was carried out at a single concentration of hapten (5×10^{-4} M). Failure of inhibition at this level has been observed with only five of eighty-five sera. In nearly all instances red cells were prepared by incubation with penicillin at pH 8.6 in barbital buffer.

uct had 3 mc. per milligram protein and 99.6 per cent of the radioactivity was precipitable by TCA. After reaction with 0.5 mM penicillenic acid and dialysis, 95 per cent of the radioactivity was precipitable by a gamma globulin fraction of rabbit antipenicilloyl antibody (rabbit anti-PNO) in the presence of carrier penicilloyl–egg albumin. Radioactivity was counted in a Packard automatic well-type scintillation counter at an average background of 58 c.p.m.

RESULTS

Screening of human sera with regard to hemagglutination titer and hemagglutination inhibition

Of the 114 sera from persons with a history of penicillin allergy or cutaneous reactivity to penicilloyl-polylysine, 65 per cent had hemagglutination titers of 1:16 or higher with cells prepared by incubation with penicillin in barbital, pH 8.6 (Table I). In the "normal" population (no history of allergic reaction and negative skin test), titers of similar magnitude (1:16 or higher)* were encountered in six of sixty-eight, or 9 per cent. Hemagglutination inhibition by penicillin derivatives could be readily demonstrated with these six sera. Significant hemagglutination titers (1:16) correlated well with cutaneous reactivity to penicilloyl-polylysine, whether or not the subject had a history of penicillin allergy (Table I). However, eight subjects with negative skin tests to penicilloyl-polylysine had hemagglutination titers of 1:16 or higher. The converse was also observed: Seventeen subjects with positive cutaneous reactions to penicilloyl-polylysine had hemagglutination titers below 1:16.

Table II gives the results of screening hemagglutination inhibition of a variety of penicillin derivatives by gamma globulin fractions of human sera. On this basis, twenty-two sera were selected for more detailed analysis.

Detailed hapten-inhibition studies with human globulin fractions

Gamma globulin fractions were routinely prepared in order to circumvent nonspecific binding of hapten by serum albumin.

On the basis of these studies, we determined the existence of six more or less distinct inhibition patterns (Table III).

Pattern A. The common pattern (of about two thirds of gamma globulin fractions studied and three fourths or more of all sera) is represented by the results with Ru (Table III). D-α-Benzyl-PNO-AC (IVa) was the most effective inhibitor. Complete inhibition at all or nearly all dilutions of gamma globulin was seen with IVa at concentrations in the range of 1 to 5 $\times 10^{-5}$ M. The effectiveness of other haptens in relation to D-α-benzyl-PNO-AC (IVa) with other gamma globulin fractions of this group was similar to that with Ru, except that PNCE-MB (X) was usually not inhibitory, even at concentration of 500 or 1,000 mμmoles per milliliter. In some instances, IVa, muta-benzyl-PNO-AC (IVc), and D-α-benzyl-PNO-benzylamide (V) appeared equal in their inhibitory activities.

Pattern B. In the second most common inhibition pattern, penicilloyl deriva-

*The choice of 1:16 as a significant titer is an arbitrary one, based on the relative titers obtained with sera from "normal" and penicillin-allergic subjects. Even at a titer of 1:8, however, hapten inhibition by D-α-benzyl-PNO-AC (IIIa) could frequently be demonstrated.

Table III. *Representative hemagglutination-inhibition patterns with gamma globulin fractions from subjects allergic to penicillin compared to patterns with globulin fractions from rabbit sera of known specificity*

*Haptens used for inhibition**	*Hapten concentration required for inhibition†*						
	Ru‡	*Av*	*Wi*	*Sa*	*Ed*	*Rabbit anti-PNO§*	*Rabbit anti-PNCE*
Benzylpenicillin (I)	500	75	60	300	> 500	1,500	50
6-APA§ (II)	500	500	> 500	50	> 500	> 1,500	> 1,500
D-α-Benzyl-PNO-AC (IVa)	10	125	> 500	150	> 500	30	300
Muta-benzyl-PNO-AC (IVc)	30	–	> 500	300	> 500	15	300
Benzyl-PNO-benzyl-amide (V)	30	–	> 500	150	–	50	300
Allylthiomethyl-PNO-AC (VI)	50	–	> 500	150	–	1,500	> 1,500
Benzylpenicilloate (VII)	50	–	> 500	150	> 500	150	1,500
D-Benzylpenilloate (IXa)	> 500	60	6	100	> 500	> 1,500	300
PNCE-MB (X)	300	15	30	500	> 500	> 1,500	15
PNCE-NES (XI)	300	15	30	–	> 500		15
Penamaldate-AC (XIII)	30	15	100	100	50	50	100
Penicillamine-MB (XV)	> 500	> 500	> 500	> 500	> 1,000	> 1,500	> 1,500
Penilloic-MB (XVI)	> 500	–	10	–	> 500		500
8-OH-Penillic (XVII)	> 500	–	> 500	30	> 500		
Penicic (XVIII)	> 500	–	> 500	20	> 500		
Penaldate-AC (XIX)	30	125	300	300	> 500		
7-ACA (XXIV)	> 500	–	> 500	> 500			
Poly-6-APA (XXVI)‖	150	–	> 500	10	> 500		

*Cells were prepared for agglutination by incubation with penicillin at pH 8.6.

†Hapten concentrations are expressed as the lowest concentration in millimicromoles per milliliter which gave marked to complete inhibition of agglutination.

‡The reciprocal of the highest dilution of gamma globulin giving agglutination and the dilution at which inhibition was studied (in parentheses) were as follows: Ru, 256 (32); Av, 64 (4); Wi, 1,024 (32); Sa, 512 (64); Ed, 256 (32); rabbit anti-PNO, 1,024 (64); rabbit anti-PNCE, 256 (64).

§For abbreviations see appropriate sections in *Materials and methods.*

‖The molarity of XXVI is calculated on the basis of its mean residue weight.

tives (IVa, IVb, IVc, and V), penicillenate derivatives (X, XI, and XII), D-benzylpenilloate (IXa), penamaldate-AC (XIII), 2-benzyl-4-hydroxymethylene-5-oxazolone (XXI), and benzylpenicillin (I) all inhibited at concentrations of 3×10^{-4} M and below.* (The relative order of effectiveness varied, but

*The penicillenate haptens PNCE-MB (X) and PNCE-NEC (XI) had a 280 mμ absorbance in excess of the reading at this wavelength due to penicillenate alone, indicating penamaldate contamination.[2, 18] The preparations varied in penamaldate content, however, and the relative inhibitory activity of the derivatives with various globulin fractions did not seem proportionate to the amount of penamaldate present. Moreover, the phenylmercurimercaptide of penicillenate (PNCE-PM, XII), which appears to have little if any penamaldate contamination, and 2-benzyl-4-hydroxymethylene-5-oxazolone (XXI), which contains the cyclic portion of the penicillenate molecule, appeared to parallel PNCE-NES and PNCE-MB in their inhibitory activities with human sera. The penamaldate-AC (XIII) derivative was in some cases a nearly equal and in others a less effective inhibitor than the penicillenate derivative. Therefore, the possibility that inhibition by PNCE haptens was primarily the result of penamaldate contamination seems unlikely.

Table IV. *Representative hemagglutination-inhibition patterns of gamma globulin fractions from rabbits immunized with benzylpenicillin**

	Hapten concentration required for inhibition† (rabbit No.)			
Haptens used for inhibition	*4*	*6*	*7*	*8*
Benzylpenicillin (I)	1,000	–	–	3,000
D-α-Benzyl-PNO-AC‡ (IVa)	30	100	5	30
Muta-benzyl-PNO-AC (IVc)	100	30	5	100
PNCE-MB (X)	300	60	30	> 1,000
Penamaldate-AC (XIII)	30	100	30	100
Penilloate (IXa)	300	100	30	> 1,000
Penicillamine-MB (XV)	> 1,000	> 1,000	> 1,000	> 1,000
Penillate (VIII)	1,000	–	–	> 1,000

*Rabbit antisera were prepared as described in *Materials and methods.*

†Hapten concentrations are expressed as the lowest concentration in millimicromoles per milliliter which gave marked to complete inhibition of agglutination.

‡For abbreviations see the first footnote in the text and *Materials and methods.*

that given previously represents an average.) This pattern, which occurred with about one of every five sera, is represented by Av (Table III).

Pattern C. Two sera have given hemagglutination reactions that were inhibited by penicillenate (X, XI, and XII) but not by penicilloyl (IVa, IVb, and IVc) haptens. With only one of these was the titer high enough (1:512) to permit detailed inhibition studies (Wi, Table III). Various penicilloyl haptens were completely ineffective as inhibitors at concentrations of 1×10^{-3} M, whereas D-penilloate (IXa), D-β-penilloate (IXb), penicillenate (X, XI, and XII), 2-benzyl-4-hydroxymethylene-5-oxazolone (XXI), penamaldate (XIII), and penicillin (I) were all notably inhibitory at concentrations below 1×10^{-4} M. Isopenillic acid (XX) and penillic acid (VIII) were inhibitory at 2×10^{-4} M.

Pattern D. A fourth type of pattern, thus far found with three globulin fractions, is represented by Sa (Table III); 6-APA (II) and penicic acid (XVIII), which correspond to deacylated penicillin (I) and penicilloic acid (VII), respectively, were more effective inhibitors than various penicilloyl derivatives, benzylpenicillin, and penicillenate-mercuribenzoate. Poly-6-APA (XXVI) and 8-hydroxypenillic acid (XVII) were also excellent inhibitors, whereas 7-ACA (XXIV) was a poor inhibitor.

Pattern E. A fifth pattern, limited so far to one serum only (Ed, Table II), shows no effective hapten inhibition except with penamaldate-AC (XIII).*

Two other sera studied were not inhibited by any hapten at low concentration, but there was too little serum for detailed evaluation with all penicillin derivatives, and penamaldate inhibition was not studied.

Pattern F. A sixth type of pattern in which the muta-benzyl-PNO-AC (IVc)

*The penamaldate derivative used for inhibition, penamaldate-AC (XIII), is not ideal since it is not entirely stable for long periods and any free mercuribenzoate liberated from the complex might directly interact with antigenic groups on the red cell, thus interfering with agglutination (see below). However, with gamma globulin fractions of the Ed serum, the penamaldoyl-mercuribenzoate derivative (XIII) was a substantially better inhibitor than free mercuribenzoate, and other mercuribenzoate mercaptides (e.g., X, XIV, XV, and XVI) were noninhibitory at concentrations one hundred fold higher than the level at which the penamaldoyl derivative (XIII) caused inhibition of hemagglutination.

was a better inhibitor than the D-α is shown especially well by the serum of Ne, which is described in more detail in Table V and not included in Table III. With the Ne globulin fraction, muta-PNO-Ac (IVc) was inhibitory at 2×10^{-5} M, whereas an 8×10^{-5} M concentration of D-α-benzyl-PNO-AC (IVa) was required for similar inhibition. Three other globulin fractions were inhibited by IVa at two to three times the concentration required with IVc.

Studies with animal sera

The antipenicilloyl globulin fraction (rabbit anti-PNO) obtained after immunization with penicilloyl bovine gamma globulin contained 0.4 mg. of specific antibody per milliliter and had a hemagglutination titer of 1:1,024. The most effective inhibitors were the mutarotated penicilloyl-aminocaproates (IVb and IVc) (Table III), an expected finding since penicillenate was used to prepare the immunizing antigen (see *Discussion*). The difference between D-α (IVa) and mutarotated (IVc) penicilloyl was slight but consistent on repeated runs.* Penicillenate-mercuribenzoate (X) was a poor inhibitor at concentrations below 1×10^{-3} M.

The antipenicillenate globulin fraction contained 0.7 mg. specific antibody per milliliter by precipitin analysis and had a hemagglutination titer of 1:256. Penicillenate-mercuribenzoate (X) was a much more effective inhibitor of hemagglutination than the penicilloyl-α-amides (Table III, rabbit anti-PNCE).

The relative effectiveness of individual haptens as inhibitors of hemagglutination of rabbit sera (anti-PNO and anti-PNCE) correlated well with their activities in prevention of precipitation as defined by the quantitative precipitin reaction. With both systems, precipitation with specific antigen at equivalence quantitatively removed hemagglutinating activity from the supernatant.

Immunization of rabbits with penicillin produced antisera the agglutination reactions of which were inhibited most effectively by penicilloyl-α-amides. Of the eight antisera evaluated, the D-α-benzyl-PNO-AC (IVa) was a better inhibitor than the mutarotated products (IVb and IVc) with five antisera, two to four times higher concentrations of the latter being required to produce inhibition comparable to the D-α enantiomorph (represented by rabbits 4 and 8, Table IV). However, one of the rabbit antisera was inhibited more effectively by mutarotated penicilloyl (IVc) (Table IV, rabbit 6, comparable inhibition at one third the concentration), and with two others the mutarotated penicilloyl was equivalent to D-α-penicilloyl (Table IV, rabbit 7). With two sera, D-benzylpenilloate (IXa) and PNCE-MB (X) inhibited hemagglutination at concentrations below 1×10^{-5} M but four other sera were not inhibited at concentrations below 3×10^{-4} M.

Hemagglutination with penicillenate-treated cells

Treating red cells with penicillenic acid rather than penicillin also gave positive hemagglutination reactions with human and animal sera. The titers with human sera were usually one to several dilutions lower than titers with

*In comparing D-α and mutarotated penicilloyl (IVa and IVc) as inhibitors, we have routinely repeated the hapten-inhibition titration at least three or four times to be certain that results were consistent.

cells prepared by treatment with penicillin in the usual manner (see for example Table V; Sm, Ge, and Ru). Four human sera with higher agglutination titers with penicillenate-treated red cells than with cells prepared in the usual fashion were identified. The most striking human serum in this group (see *Pattern F,* Ne) had a titer of only 1:4 with penicillin-treated cells but 1:64 with cells that had been reacted with penicillenic acid (Ne, Table V). With globulin fractions of each of these four sera the mutarotated benzylpenicilloyl-aminocaproates (IVb and IVc) were substantially more effective inhibitors of hemagglutination than the corresponding D-α derivative. Penicillenate haptens were relatively poor inhibitors. Similar correlations were made with seven animal sera. It was found that where the D-α-PNO-benzyl-AC (IVa) was a better inhibitor than muta-PNO-benzyl-AC (IVc), the penicillin-treated cells gave a higher titer than those prepared with penicillenate. Where IVc was the better inhibitor, the penicillenate-treated cells were more effective in agglutination. Penicillin-treated cells subsequently incubated with mercuribenzoate and washed were similar in reactivity to penicillenate-treated cells (Table V).

Coprecipitation of radioactive antigen by human globulin fractions

Gamma globulin fractions of thirty-one human sera (six normal and twenty-five with penicillin allergy) were evaluated for the ability to cause coprecipitation of I^{131}-labeled penicilloyl bovine serum albumin when a globulin fraction of a rabbit antiserum specific for human gamma globulin was added. With eight of the globulin fractions from penicillin-sensitive subjects, more than 40 per cent of the labeled antigen was specifically precipitated (as judged by hapten inhibition). Precipitation of radioactivity by globulin fractions of control sera in all cases was less than 10 per cent of the total antigen added. There was relatively little correlation between the extent of specific precipitation of

Table V. *A comparison of the hemagglutination reactions of sera specific for penicilloyl: The effect of antibody stereospecificity*

	Best steric inhibitor		*Hemagglutination titer*					
Sera tested	*D-α-IVa*	*Muta-IVc*	*PN cells* (pH 8.6)*	*PN cells* (pH 7.4)*	*PNCE cells†*	*PN cells + MB‡ (pH 8.6)*	*PN cells + MB‡ (pH 7.4)*	*PNCE cells + MB‡*
Rabbit anti-PNO§		+	1,024		2,048	2,048		2,048
Rabbit anti-PN‖	+		256	–	64	64	–	32
Human Ne		+	4	4	64	32	4	64
Human Sm	+		512	16	32	64	4	32
Human Ge	+		1,024	32	128	128	4	128
Human Ru	+		512	8	256	256	4	256

*PN cells were prepared by incubation with penicillin at pH 8.6 or 7.4 as indicated.

†PNCE cells were prepared by incubation with penicillenic acid (III) at pH 8.6.

‡Incubation of cells with mercuribenzoate (MB), 4×10^{-5} M, was carried out at pH 8.2 for 30 minutes at room temperature. For further details see *Materials and methods.*

§For method of preparation of this serum see *Materials and methods* and Table III.

‖An antiserum obtained after immunization of a single rabbit with penicillin (see Table IV, rabbit No. 4).

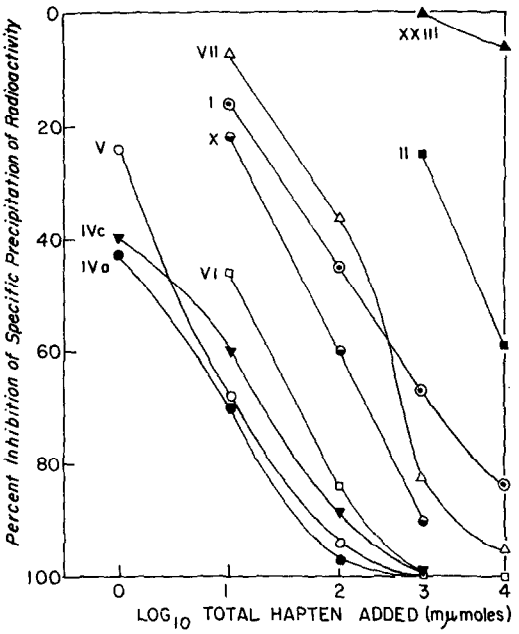


Fig. 2

Hapten inhibition of coprecipitation of I^{131} penicilloyl–bovine gamma globulin. In duplicate tubes equal volumes (0.05 ml.) of hapten in phosphate-saline, a gamma globulin fraction of human serum T. S. (allergic to penicillin), and I^{131}-labeled penicilloyl–bovine gamma globulin (0.04 μg) (see *Materials and methods*) were incubated for 15 minutes at 37° C. and 15 minutes at 4° C. An excess of a gamma globulin fraction of rabbit antihuman globulin (0.80 ml.) was added and, after 15 minutes at 37° C., the tubes were centrifuged at 4° C. and an aliquot of the supernatant solution counted to a minimum of 14,000 counts above background. In the absence of hapten, 78 per cent of the total radioactivity was precipitated. With normal human gamma globulin fractions, the amounts of radioactivity precipitated were 6.2, 7.1, and 7.6 per cent. The average difference of 71 per cent is presumed to represent specifically precipitated radioactivity, and the average value for the three normal sera (7 per cent) is used to represent 100 per cent inhibition of specific precipitation. The haptens at concentrations as high as 3×10^{-2} M had no effect on nonspecific precipitation of radioactivity.

The addition of human serum albumin to concentrations as high as 10 mg. per milliliter did not increase precipitation of radioactivity in tubes containing globulin fractions from normal subjects.

radioactivity and the hemagglutination titer. Detailed evaluation of hapten inhibition of antigen coprecipitation was carried out with two globulin fractions, each with hemagglutination inhibition pattern A. The results with the globulin fraction of T. S. (Fig. 2) are similar to those obtained with the other globulin fraction evaluated in detail. Unfortunately, the globulin fractions with more unusual hemagglutination patterns (Table III: Av, Wi, Sa, Ed) caused relatively little coprecipitation of radioactive antigen above control values, precluding studies of a similar nature with these fractions.

Effects of antihuman globulin and 2-mercaptoethanol on hemagglutination titer

The effects of incubation of human sera with 0.1M 2-mercaptoethanol for 2 hours at neutral pH were evaluated with twenty-two sera (represented by

the eight sera in Table VI). With eighteen sera, 2-mercaptoethanol inhibited the ability of sera to mediate hemagglutination (a twofold or greater decrease in titer). The titers of these sera were not augmented by incubation of cells with antihuman globulin. The most striking exception was serum Ca, which had a high titer by the Coombs technique and was unaffected by the mercaptan. Preliminary results with Sephadex G-200 chromatography were consistent with the localization of hemagglutinating activity, which is readily inhibited by mercaptan to the macroglobulin fraction (Table VI, footnote). With serum Ca the results of Sephadex fractionation suggested that the immune globulins involved were 7S. It was impossible to show that the Ca serum (without antihuman globulin) could inhibit hemagglutination mediated by other sera of high titer. It was also impossible to show that sera which had lost hemagglutinating activity due to incubation with 2-mercaptoethanol could inhibit hemagglutinations mediated by sera retaining activity in the presence of the mercaptan.

DISCUSSION

Preparation of erythrocytes for hemagglutination

In preparing red cells for hemagglutination, the usual procedure was the incubation of red cells with benzylpenicillin at neutral pH either in plasma or saline followed by saline washes. It has generally been assumed that cells are "sensitized" for hemagglutination by adsorption of penicillin to the cell wall,[28] although there is some evidence contradictory to this assumption.[3, 29] On the

Table VI. *Effects of antihuman globulin and 2-mercaptoethanol treatment on hemagglutination titers of human sera*

Serum	*Hemagglutination titer**			
	Untreated serum		*Serum after 2-mercaptoethanol*	
	Without antihuman globulin	*With antihuman globulin*†	*Without antihuman globulin*‡	*With antihuman globulin*§
Ga	128	No change	4	–
Di	64	No change	4	–
Sa‖	512	No change	< 4	–
Ts	512	No change	4	4
Mo‖	64	No change	4	8
Li	64	128	32	64
Ge	512	–	256	–
Ca¶	4	256	8	256

*Cells were prepared by incubation with penicillin at pH 8.6.

†Cells were incubated with various dilutions of human serum with and without hapten for 30 minutes, washed four times with phosphate-saline, and resuspended in a diluted rabbit serum specific for human gamma globulin. Increases in titers following addition of the rabbit antiserum did not occur if D-α-benzyl-PNO-AC at a concentration of 5×10^{-4} M was present during the initial incubation (cells with human serum).

‡Sera were incubated in phosphate-saline at a dilution of 1:2 in 0.1M 2-mercaptoethanol for 2 hours at room temperature before dilution and incubation with red cells.[26]

§Sera were treated with 2-mercaptoethanol as described by Leddy and colleagues,[27] dialyzed against phosphate-saline, and cleared before incubation with red cells (in order to avoid nonspecific agglutination).

‖On Sephadex G-200 chromatography (0.25M NaCl), hemagglutinating activity was in the first, or presumed high-molecular weight, fraction.

¶On Sephadex G-200 chromatography, hemagglutinating activity (with antihuman globulin) was in the second, or presumed 7S, fraction.

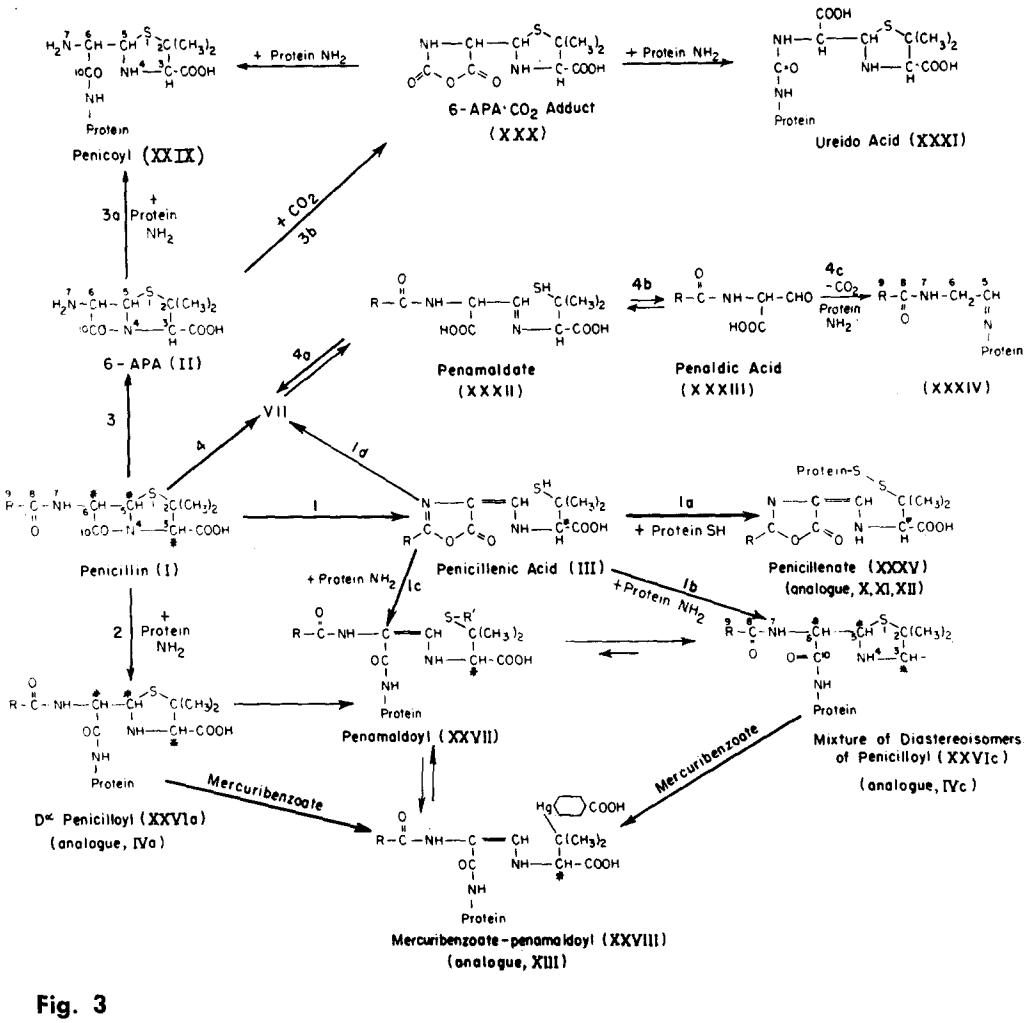


Fig. 3

Proposed pathways for formation of antigen in penicillin allergy. For derivatives represented by Roman numerals see Fig. 1 and the *Materials and methods* and *Discussion* sections. *VII* is penicilloic acid; *XXVIII* is shown in reference to the discussion of the effect of mercuribenzoate on penicillin-treated red cells. *Protein–NH* refers to an ϵ-amino group of a protein.

other hand, the "sensitization" of the cells by exposure to penicillin might involve the formation of penicilloyl and other penicillin derivatives linked covalently to red cell amino acid residues. In this case, it should be possible to direct the formation of the covalently bound derivatives, thereby preparing cells more effective in hemagglutination.

The mechanisms by which red cell–bound penicilloyl might be formed are shown in Fig. 3 (pathways *1* and *2*). The direct reaction of penicillin with protein amino groups (pathway *2*) is much more rapid in alkaline solution, where a relatively high proportion of protein amino groups are uncharged, than it is at neutrality. Penicillenate formation from penicillin (pathway *1*) is favored at neutral or acid pH. The reaction of penicillenate with protein amino and

sulfhydryl groups (Fig. 3, pathways *1a, 1b,* and *1c*) takes place readily at neutral pH, although it is favored in weakly alkaline solution.

In an attempt to evaluate the possibility that pathway *2* is involved in the sensitization of cells, red cells incubated with penicillin over a pH range from 6.0 to 8.6 were compared with regard to titers obtained with sera of thirty subjects having penicillin allergy.[30, 31] Cells prepared at pH 6.0 usually had little or no activity, even with sera of high titer (1:64 or higher), although with one serum a titer of 1:64 was obtained. Cells prepared by incubation with penicillin at pH 8.6 in barbital buffer were equivalent with some sera to cells treated in the conventional manner with penicillin (at neutral pH in donor plasma or in phosphate-saline) or, more frequently, were agglutinated at a substantially higher dilution of serum (often a fourfold or greater increase in titer; see for example Table V: Ru, Sm, and Ge). The increased sensitivity afforded by the use of alkaline pH during the incubation of cells with penicillin was quite useful in our study.

Two other observations also indicate the presence of covalently bound penicillin derivatives (presumably primarily penicilloyl) on the red cell. First, in accord with data of others,[3, 29] there was no fall in hemagglutination titers with standard antisera, even though cells were washed twenty to twenty-five times with large volumes of phosphate-saline over a 1 week period. Second, studies with tritium-labeled penicillin indicated the presence of "firmly bound" penicillin derivatives on erythrocytes following incubation of cells with H^3-labeled penicillin in barbital buffer.[31]

A second method of encouraging the formation of covalent bonds between penicillin derivatives and the red cell is by incubation of cells with penicillenate (Fig. 3, pathway *1*). Studies by deWeck[3] and Fischer and deSimone[29] have indicated that "sensitization" of red cells by penicillin is caused by intermediary formation of penicillenate, and deWeck has reported that penicillin- and penicillenate-treated cells ordinarily give similar hemagglutination titers with rabbit antisera to penicillin. In our experience, there are notable exceptions. As shown in Table V, some sera have much higher titers with the penicillenate-treated cells, whereas with other sera, the cells that reacted with penicillin at pH 8.6 more effectively promoted agglutination. From a group of twenty-seven human sera of low titer (1:4 or less) as determined with penicillin-treated cells, the titer of four was 1:16 or higher with penicillenate-treated cells.[31]

With every serum with which penicillenate-treated cells have given a higher titer than penicillin-treated cells, penicilloyl-aminocaproate in the form of a mixture of diastereoisomers (IVb and IVc) inhibited hemagglutination more effectively than D-α-benzyl-PNO-AC (IVa); by contrast, the D-α derivative has been a more effective inhibitor with sera which had higher titers with the penicillin-treated cells (see examples in Table V).

The interpretation that the differences between the hemagglutinating activities of penicillin-treated and penicillenate-treated cells are due primarily to differences in the steric configurations of red cell–bound penicilloyl is supported by the results of incubating cells with mercuribenzoate. The incubation of red cells with mercuribenzoate provides a means of epimerizing penicilloyl sub-

stituents in the D-α configuration (XXVIa) to a random mixture of penicilloyl diastereoisomers (XXVIc). The mercuribenzoate incubation lowered the effectiveness of penicillin-treated cells in promoting agglutination with sera of specificity for D-α penicilloyl (IVa) and increased the titers obtained with sera having specificity for the random mixture of penicilloyl diastereoisomers (IVc). As expected, mercuribenzoate treatment of cells prepared by incubation with penicillenate had no effect on hemagglutination titers obtained with these cells.

Antibody specificities in penicillin allergy

In a study of the antibody specificities of individual sera by means of hemagglutination inhibition, evaluation of the relative effectiveness of various haptens as inhibitors is most readily made by comparing the minimal concentrations required to inhibit agglutination. Since the ratios of antigen and antibody undoubtedly vary in individual titrations and since antibody is heterogeneous in its binding affinity for antigen, the use of an end-point assay (as opposed to various degrees of partial inhibition) is subject to theoretical objections. However, with rabbit antisera specific for penicilloyl and penicillenate, the relative order of effectiveness of haptens as inhibitors of hemagglutination was entirely consistent with the results of hapten inhibition of precipitation. Moreover, it has been found with measurements of the antibody-hapten interaction by other techniques that the heterogeneity index of a given antiserum for a series of related haptens is about the same and that it is not changed to any great extent by the relative amounts of antigen and antibody present.[32, 33] In accord with these observations, we have found that the relative order of effectiveness of various haptens as inhibitors of hemagglutination with a given globulin fraction correlated well with their relative inhibitory activities at greater dilutions of the same globulin fraction. One can therefore compare the various haptens with one another using only one or two dilutions of a given globulin fraction. In comparing two different sera with apparently the same antibody specificity (for example, two sera inhibited effectively only by penicilloyl haptens), the concentration of inhibitor needed for complete inhibition varied, as expected, over at least a twentyfold range.

In recent articles, considerable evidence has accumulated implicating the penicilloyl group in penicillin allergy.[1-3, 5, 29, 34] In our study, the results of hemagglutination inhibition with sera from humans and rabbits immunized to penicillin furnished further evidence of the importance of the penicilloyl group.* Of 114 sera from subjects with a history of penicillin allergy, eighty-five had titers of 1:16 or higher (combining results with penicillin-treated cells, penicillenate-treated cells, and penicillin-treated cells in combination with antihuman globulin).[31] D-α-Benzyl-PNO-AC (IVa) inhibited hemagglutination at levels of 5×10^{-4} M or lower in all but five. Although the penicilloyl group was found to be the dominant antigenic determinant in hemagglutination reactions produced by these sera, the diversity of antibody specificities was impressive. Among

*At the same meeting where a brief report of these observations was made,[30] VanArsdel and Tobe also presented evidence for penicilloyl specificity in human penicillin allergy from results of a hemagglutination assay[35]; their results were reported in more detail during the preparation of this article.[36]

diastereoisomers of benzylpenicilloyl-aminocaproate, the optimal inhibitor varied; of twenty-five globulin fractions evaluated, the D-α penicilloyl derivative (IVa) more effectively inhibited agglutination in sixteen. In two a random mixture of penicilloyl diastereoisomers (IVc) was the better inhibitor, and in seven it was impossible to tell them apart. Moreover, some sera clearly contained antibodies specific for penicillin derivatives other than penicilloyl, occasionally in the apparent absence of penicilloyl-specific antibody.

In early studies with penicilloyl derivatives it was assumed that protein-bound penicilloyl was formed by a preliminary rearrangement of penicillin to the highly reactive penicillenate molecule (III), which in turn coupled with protein amino groups to form a penicilloyl amide (Fig. 3, pathway *1*).[1, 2] The fact that penicilloyl in the D-α configuration (Fig. 3, *XXVIa*) was usually the most effective inhibitor with human sera could be considered as favoring pathway *2*, in which penicillin reacts with protein in vivo without intermediary formation of penicillenate.* While we favor the interpretation that pathway *2*, as well as pathway *1*, contributes to formation of antigen, no really firm conclusions are possible at this time. With sera such as Ed, the hemagglutination assay used may have failed to detect low concentrations of antibodies to steric configurations of penicilloyl other than D-α; on the other hand, the presence of such antibodies in serum Ne could be due in part to the mutarotation of protein-bound D-α-penicilloyl (XXVIa) in vivo to form XXVIc as well as to the formation of XXVIc from penicillenate. Experience to date with rabbit antisera indicates that the configuration of penicilloyl on the immunizing protein conjugate is reflected in the hemagglutination-inhibition pattern. Further study of this is needed, however. Moreover, such results in experimental animals by use of heavily substituted heterologous proteins do not necessarily indicate what might be observed in man, in whom the immunization would be to lightly substituted autologous proteins.

The results of detailed analysis of hapten inhibition of hemagglutination and coprecipitation of radioactive antigen with human globulin fractions apparently specific for penicilloyl (pattern A, represented in Table III by Ru

*Preliminary studies in experimental animals are consistent with but by no means conclusive for this viewpoint; a variety of acid-stable penicillins have been found to induce a significant antibody response as judged by hemagglutination, despite the fact that their rates of degradation to penicillenate at neutral pH appear to be much less than that of benzylpenicillin.[31] For example, α-phenoxyethylpenicillin, which develops absorbance at 322 mμ at about 1 per cent the rate of benzylpenicillin at pH 7.4 (37° C.), but which reacts with the α-amino group of glycylglycine at pH 7.8 about 50 per cent as fast as benzylpenicillin, is capable of effecting an antibody response specific for D-α-penicilloyl on administration in Freund's adjuvant to rabbits. deWeck also has commented on a lack of correlation between rate of rearrangement to penicillenate and allergenicity among penicillin analogues, suggesting as one possible explanation that penicillenate might not be an obligatory intermediate in formation of antigen.[37] One of the problems in studies of this kind, however, is the presence of impurities in many of the semisynthetic penicillins. Levine has found that mutarotated penicilloyl (IVc) was a slightly better inhibitor of precipitation than D-α-penicilloyl (IVa) using a pooled globulin fraction from rabbits immunized with penicillin.[2] In our study, the D-α derivative was, on the average, a slightly better inhibitor of hemagglutination than epimerized penicilloyl (IVb, IVc). However, one of our rabbit antisera to penicillin was specific for mutarotated penicilloyl, and it is entirely possible that the difference in the two studies is due to random sampling of sera. The difference in the two assays employed may also exert some influence.

and in Fig. 2) were very similar to those obtained by hapten inhibition of precipitation by use of globulin fractions from rabbits with penicilloyl-specific antibodies.[18, 38] In the induction of antibodies specific for penicilloyl the required protein conjugate would be formed by the coupling of penicilloyl to protein lysyl residues (in Fig. 3, *XXVIa* and *XXVIc*, the protein NH group is at the end of the alkyl side chain of lysine). The near-equivalence of D-α-benzyl-PNO-AC (IVa), which has all the carbons corresponding to the alkyl side chain of lysine, and benzyl-PNO-benzylamide (V), which does not, indicated that residues 1 to 10 in Fig. 1 *(IV* and *VII)* and the NH-CH_2 moiety to which C_{10} is attached account for most of the energy of interaction between antigen and antibody. The less effective inhibition by allylthiomethyl-PNO-AC (VI) may be due to a contribution by the benzyl side chain to specific binding or due to steric hindrance of binding by the allylthiomethyl moiety. The relatively weak inhibitory activity of penicilloate (VII) is related to the negative charge at the C_{10} position at neutral pH as well as to the absence of the amide bond at C_{10}.

The weak inhibitory activity of 6-APA with globulin fractions from subjects with penicillin allergy (usually at 1×10^{-3} M or higher) may be ascribed to some structural similarity between 6-APA and other penicillin derivatives which are much better inhibitors. The identification of three sera (Sa, Table III) with greater specificity for 6-APA (II) and its derivatives (XVII, XVIII, and XXVI) than for other penicillin derivatives is of considerable interest. In this case, the formation of antigen would presumably involve deacylation of benzylpenicillin in vivo (Fig. 3, pathway *3*), followed by the reaction of 6-APA or a 6-APA derivative with protein.* That 6-APA can be formed from penicillin in vivo is shown by the presence of 6-APA in the urine of subjects and animals receiving benzylpenicillin.[39] Once formed, 6-APA is a potent antigen.[37, 40, 41] Although the mechanism of the reaction of 6-APA with protein is uncertain, there are at least two possibilities. 6-APA can react with its own weakly basic amino groups at pH 6 to 7 to form a polymer.[23] This suggests that 6-APA might react with protein amino groups to yield penicoyl (*XXIX*, Fig. 3) or its polymer in a manner analogous to the formation of protein-bound penicilloyl directly from penicillin in alkaline solution (Fig. 3, pathway *3a*, as compared with pathway *2*). Another possibility would be through the formation of an adduct between 6-APA and CO_2[21, 42] (Fig. 3, pathway *3b*, XXX). Derivative XXX is an *N*-carboxy-α-amino acid anhydride analogous to derivatives used to prepare poly-α-amino acids.[42] It is highly reactive under physiologic conditions and can form both penicoyl (XXIX) and a ureido acid (XXXI) on reaction with protein amino groups or degrade further to form 8-hydroxypenillic acid (XVII).[23, 43]†

*Cleavage of protein-bound penicilloyl at the N-7, C-8 amide bond (Fig. 3, *XXVIc*) would be another possibility.

†Fischer and deSimone noted that red cells incubated with 6-APA which had been dissolved in bicarbonate buffer had higher hemagglutination titers with antisera to penicillin than cells in which the incubation was carried out in phosphate buffer at neutrality and the preliminary addition of $NaHCO_3$ omitted.[29] They attributed this effect to the formation of the 6-APA-CO_2 adduct in bicarbonate. Another less likely possibility is that pathway 3a was involved and that the higher pH in the presence of bicarbonate favored the reaction by increasing the proportion of unchanged red cell amino groups. Since racemization should be minimal or absent in the formation of the 6-APA-CO_2 adduct, it would not be possible to distinguish pathway 3a from 3b by an examination of the stereo-specificity of the antibodies formed.

If penicoyl (XXIX) is the protein-bound 6-APA derivative giving rise to antibody formation, the ideal hapten for inhibition would be penicoyl-aminocaproate (Fig. 1, *IV,* in which 7 is NH_2 and 8 and 9 are deleted), which has the same relation to 6-APA as penicilloyl-aminocaproate (IV) has to penicillin. Preliminary results with this derivative indicate that it is a less effective inhibitor than poly-6-APA (XXVI), and the antigenic determinant here may be poly-6-APA itself. Poly-6-APA also is a very effective inhibitor of hemagglutination and precipitin reactions of rabbit antisera to 6-APA. Another possibility is that penicoyl (XXIX) becomes substituted at N-7 by acetyl or some other acyl group following its attachment to protein.

In addition to three sera for which poly-6-APA (XXVI) was the best inhibitor, with two other human globulin fractions, XXVI inhibited hemagglutination at 5×10^{-5} M, which is only a three- to fivefold higher concentration than the level of IVa required for corresponding inhibition, and 6-APA inhibited more effectively than penicillin.

With the human globulin fraction Ed (Table III) penamaldate-AC (XIII) was the best inhibitor of hemagglutination. This suggests that antibodies were specific for penamaldoyl (Fig. 3, *XXVII,* in which oxidation of S stabilizes the molecule), which could arise either by pathway *1* or *2*. Another possibility is that penamaldate (XXXII) becomes bound to protein through formation of a mixed disulfide bond with protein half-cystine residues in a manner analogous to the conjugation of penicillenate to protein (XXXV). In regard to inhibitions by XIII with other globulin fractions, cross-reactivities with other structures such as XXVIc (Fig. 3) may be partly involved. However, the level of XIII required for inhibition is often comparable to that of penicilloyl-aminocaproate (IVc). This suggests that antibodies to penamaldate and/or penamaldoyl (XXVII) are relatively common in penicillin allergy.

With the human globulin fraction Wi (Table III) the best inhibitor of hemagglutination was benzylpenilloate (IXa and b). A mechanism by which antibodies highly reactive with this derivative might be induced is shown in Fig. 3, where penicilloate (VII) formed by reactions *4* and *1d* gives rise by reactions *4a* and *4b* to penaldic acid (XXXIII), which could form an imine derivative with simultaneous decarboxylation[44] yielding XXXIV.* The attachment of XXXIV to protein might be stabilized by resonance or by reduction at the imine bond. The postulated derivative XXXIV would contain the residues of the penilloate molecule numbered 5 to 9 (Fig. 1, *IX*). The similar inhibitory activities of D-penilloate (IXa) and D-β-penilloate (IXb) (which differ in average configuration at the asymmetric C-5) and the mercuribenzoate derivative of penilloate (XVI) (in which the thiazolidine ring of penilloate is cleaved and C-5 is no longer asymmetric) would be consistent with a determinant such as XXXIV, in which there is no thiazolidine ring and no asymmetry at C-5.†

More difficult to evaluate are human globulin fractions with inhibition pattern

*Levine has reported that benzylpenilloaldehyde, which is decarboxylated penaldic acid, induces contact skin sensitivity in the guinea pig.[45]

†Another possibility is that the product formed by cleavage of IXa between residues *1* and *5* (Fig. 1) is attached in disulfide linkage to protein. In this circumstance one might expect XVI to be the best inhibitor.

B (represented by Av, Table III) and globulin fractions from rabbits Nos. 6 and 7, immunized with penicillin (Table IV), where there was effective inhibition by penicilloyl (IV), penicillenate-MB (X), and D-penilloate (IXa).* By comparing these results with those obtained with human globulin fractions with inhibition pattern A and rabbit antisera of known specificity, it seems clear that at least two antibody specificities are involved, one of which is to penicilloyl. As to the nature of the second determinant(s), it is necessary to withhold judgment at this time. It may be that group B is heterogeneous and that penicillenate *(XXXV)* and *XXXIV* (Fig. 3) are both involved as antigenic determinants in some cases. There is the further possibility that a third structure not yet examined in detail may be involved.† It is possible that the specificities involved here are responsible for cutaneous reactivity to penicillenate–thiolated human serum albumin.[1]‡

In our study the variety of antigenic determinants involved in penicillin allergy was evident, but other reports concerning the mechanism of allergic reactions to penicillin have not dealt with this aspect to any great degree. This difference is primarily the result of our having evaluated a larger number of individual sera. The diversity of antibody specificities following penicillin administration is significant in relation to the use of penicilloyl-polylysine as a screening agent for penicillin allergy in man.[1] It seems likely that subjects with negative cutaneous reactions to penicilloyl-polylysine and positive responses to other penicillin derivatives will ultimately be identified.

That the number of antibody specificities in penicillin allergy is not primarily due to differences in preparations of penicillin used for immunization is shown by studies with the sera of rabbits immunized simultaneously with a single lot of penicillin. The variation in the spectrum of antibody specificity produced to penicillin may be mainly due to differences in the antibody response of individual animals simultaneously stimulated with several antigens in relatively low concentration. It is also possible that differences in the mode of degradation of penicillin in individual animals influence the nature of the antibody response.

*deWeck has reported that hemagglutination by a pooled rabbit antiserum to penicillin was inhibited by penicillenate at concentrations about 30 to 100 times higher than concentrations of penicilloyl derivatives required for corresponding degrees of inhibition.[3]

†The results of immunization of guinea pigs with a lightly substituted penicilloyl-polylysine prepared by reaction of penicillin with polylysine at pH 9.5 may be relevant here. With two of three globulin fractions in which hemagglutinating activity with penicillin-treated erythrocytes was noted, D-α-benzyl-PNO-AC (IVa) was inhibitory at concentrations of 2 and 3 × 10^{-5} M, whereas X and IXa inhibited in the 1 to 2 × 10^{-4} M range. This observation indicates that nonpenicilloyl substituents on the polylysine can induce an antibody response. The similarity of the inhibition pattern to that of Av and other globulin fractions with pattern B is of interest. The only group, in addition to penicilloyl, which is known to be present on penicilloyl-polylysines prepared in this manner is evidenced by its 280 mμ absorbance. In addition to structures already suggested to account for the acid-stable absorbance in this region,[1, 2] an imidazolone (as in *XXII*, Fig. 1, where the N-9 is derived from a lysine ε-amino group), an oxazolone (XXI), or an oxazole should be considered. Both XXI and a crude preparation of XXII were very good inhibitors with human globulin fractions also inhibited by PNCE-MB (X), but further evaluation is needed.

‡With four globulin fractions from subjects with cutaneous reactivity to both the penicillenate protein and penicilloyl-polylysine, penicillenate-MB (X) inhibited hemagglutination at a concentration of 2 × 10^{-4}M or below in two, at 4 × 10^{-4}M in a third, and at 1 × 10^{-3}M in the fourth. D-α-benzyl-PNO-AC was inhibitory at concentrations below 1 × 10^{-4}M in every instance.

It would have been interesting to study the extent to which 6-APA is formed from penicillin in Sa, since in many human subjects it is impossible to demonstrate urinary excretion of 6-APA following penicillin administration.[16]

Although many people with penicillin allergy have both cutaneous reactivity to penicilloyl-polylysine and sera with significant hemagglutination titers, occasionally there is no correlation between these two factors (Table I).[10, 36] This is not surprising in view of recent studies in man and in the guinea pig which indicate that cutaneous sensitization and hemagglutination are in some cases mediated by different types of immune globulins, classified as beta-2A (or gamma-1A) and beta-2M (or gamma-1M), respectively.[27, 47-49] The indication that direct hemagglutination reactions by human sera in penicillin allergy are mediated primarily by macroglobulins agrees with the earlier findings of Fudenberg and German.[7]

SUMMARY

By a modification of the technique of hemagglutination by use of cells sensitized by incubation with penicillin at pH 8.6, titers of 1:16 or higher were obtained with the majority of sera from subjects with penicillin allergy. With the increased sensitivity of the hemagglutination assay, detailed hapten-inhibition studies with many human sera were possible. The results were compared to those obtained with sera from rabbits immunized with penicillin, penicilloyl–bovine gamma globulin, and penicillenate–thiolated bovine gamma globulin.

Evidence is presented which indicates that penicilloyl in the D-α configuration is, in general, the most effective inhibitor of hemagglutination reactions mediated by sera of humans immunized to benzylpenicillin. However, with individual sera, penicillenate, penilloate, penamaldoyl, a random mixture of penicilloyl enantiomorphs, or derivatives of 6-aminopenicillanic acid may be more effective inhibitors, and a variety of combinations of antibody specificities are observed.

The fact that D-α penicilloyl is the best inhibitor among several preparations of penicilloyl diastereoisomers is discussed in relation to its possible relevance to the mechanism of formation of penicilloyl antigen from penicillin in vivo. It is suggested that protein-bound penicilloyl is formed both by direct reaction of penicillin with protein amino groups and through rearrangement of penicillin to the intermediate, penicillenic acid.

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