The infrared spectrum of this material was superimposable upon the spectrum obtained from the product of procedure A described above.

Acknowledgments.—We are indebted to Mr. D. F. Whitehead for the infrared spectra, to Mr. R. M. Downing for the elemental analysis, to Miss L. V. Latta for determination of susceptibility to penicillinase and to Miss C. Bernardi for valuable assistance in the preparation of the intermediate phthalamic acids.

N(α-D-Penicilloyl) Amines as Univalent Hapten Inhibitors of Antibody-Dependent Allergic Reactions to Penicillin[1]

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Received February 21, 1962

A series of N-(α-D-penicilloyl) amines (α-diastereoisomers) was prepared by reaction of penicillins (benzylpenicillin, dimethoxyphenylpenicillin, and allylmercaptomethylpenicillin) with various amines. Methods for the preparation of diastereoisomeric mixtures of these penicilloyl amines, and a spectrophotometric method for their quantitative assay are given. Quantitative comparative data were obtained showing the diastereoisomeric mixtures of the penicilloyl amines to be capable of specifically inhibiting the *in vivo* and *in vitro* reactions of rabbit antipenicillin antibodies with a conjugated penicillin antigen (benzylpenicilloyl-human *gamma*-globulin). These data suggest that these penicilloyl amine haptens may be useful as therapeutic agents to prevent and treat antibody-dependent allergic reactions to penicillin in man.

In previous studies on the mechanism of antigenicity of benzylpenicillin, the diastereoisomeric mixture of ε-N-(α-D-benzylpenicilloyl)-lysine groups (IIi) (which are finally formed by the reaction of benzylpenicillin with lysine ε-amino groups of tissue proteins) were identified as the major antigenic determinants responsible for allergy to benzylpenicillin.[3–5] The diastereoisomeric mixture of ε-N-(α-

(1) This investigation was supported by the U. S. Army Medical Research and Development Command, Department of the Army, under Research Grant No. DH-MD-49-193-61-G29.

(2) Special Research Fellow of the Public Health Service.

(3) B. B. Levine, *J. Exp. Med.*, **112**, 1131 (1960).

(4) B. B. Levine, *Arch. Biochem. Biophys.*, **93**, 50 (1961).

(5) B. B. Levine and Z. Ovary, *J. Exp. Med.*, **114**, 875 (1961).

D-benzylpenicilloyl)-aminocaproic acid was found to be an effective hapten inhibitor of the reaction of rabbit and human antibenzylpenicilloyl antibodies with a benzylpenicilloyl-human *gamma*-globulin conjugated antigen.[5] These observations prompted the suggestion that N-(α-D-benzylpenicilloyl) amines and amino acids might be useful as therapeutic agents to prevent and treat antibody dependent allergic reactions to benzylpenicillin.[5]

The present report is an initial investigation of this possibility. It describes the preparation of a group of N-(α-D-penicilloyl) amines and amino acids (II), a method for their quantitative assay, and a comparative study with regard to the ability of these penicilloyl amines (univalent haptens) to inhibit the reaction of rabbit antibenzylpenicilloyl antibodies with an N-α-D-benzylpenicilloyl-human *gamma*-globulin conjugated antigen *in vitro* and *in vivo*.

These new penicilloyl amines (II) were prepared in high yield by reaction of various penicillins (benzyl, dimethoxyphenyl and allylmercaptomethyl) with the amine or amino acid in aqueous solution at pH 11.5 to 12. This method has been used previously to prepare N-(α-D-benzylpenicilloyl) derivatives of ammonia, methylamine and benzylamine[6a] and would appear to constitute a general method for the preparation of N-(α-D-penicilloyl) amines. The reaction products are α-diastereoisomers as the two asymmetric carbon atoms of the β-lactam ring of the penicillins retain their configurations. The α-diastereoisomers were crystallized without difficulty as hydrated benzylamine salts. As reported previously,[6a] the α-diastereoisomers could not be crystallized as the free acids, sodium salts or hydrochloric acid salts except for N-(α-D-benzylpenicilloyl) *n*-octylamine (IIe) which crystallized from the reaction mixture as the sodium salt. Elemental analyses and physical constants of the N-(α-D-penicilloyl) amines, α-diastereoisomers are given in Table I.

The diastereoisomeric mixtures of the N-(α-D-penicilloyl) amines were prepared by incubating the α-diastereoisomers in aqueous solution at pH 5, 37°, for 3 hr., in the presence of trace cupric ion, or by refluxing in aqueous solution at pH 5 for 10 min. Partial racemization of the two centers probably proceeds through the reversible formation of N-(α-D-penamaldoyl) amines (III). This was indicated by the effect of catalytic quantities of cupric ion to markedly increase the rate of mutarotation and the appearance of new λ 285 mμ absorbing substances in the mutarotated N-(α-D-penicilloyl) amine solutions. These new substances exhibited the ultraviolet spectral characteris-

(6) "The Chemistry of Penicillin," (H. T. Clarke, J. R. Johnson and R. Roberson, editors), Princeton University Press, Princeton, N. J., 1949: (a) Chap. 18; (b) p. 427.

tics, and underwent the reactions described previously for N-(α-D-penamaldoyl) amines.[6b]

A new rapid spectrophotometric method for the quantitative assay of N-(α-D-penicilloyl) amines is given. It is based on the known reaction of N-(α-D-penicilloyl) amines with mercuric chloride in ethanolic solution to form N-(α-D-penamaldoyl) amines.[6b] It was found here that maximum stability of the λ 285 mμ penamaldate absorption peak was achieved when the penamaldate reaction was carried out in pH 9.2 carbonate buffer and with 4 to 50 *M* equivalents of *p*-chloromercuribenzoate (rather than mercuric chloride). The results were reproducible within 1.5% and as little as 5 mμmoles of penicilloyl amine could be analyzed.

The diastereoisomeric mixtures[7] of the N-(α-D-penicilloyl) amines (univalent penicilloyl haptens) listed in Table I were compared with regard to their abilities to specifically inhibit the precipitation of rabbit antibenzylpenicilloyl antibodies by an N-α-D-benzylpenicilloyl-human *gamma*-globulin conjugated antigen. The hapten inhibition methods originally described by Landsteiner[8] and placed on a quantitative basis by Pauling, Pressman and Campbell and their associates[9] were used. The inhibition curves are shown in Fig. 1 and the concentrations of the penicilloyl amine haptens which were required to achieve 50% inhibition are listed in Table I. The results in Fig. 1 showing a family of sigmoid curves are similar to the results obtained by Pauling, *et al.*, for azoprotein systems.[9] They ascribed the shape of these curves to heterogeneity of the antibody binding sites with regard to their affinities of binding to antigenic determinant groups. Inhibition by the penicilloyl haptens was specific as even higher concentrations of these haptens did not inhibit precipitation of rabbit antibovine *gamma*-globulin by bovine *gamma*-globulin. Benzylamine did not cause significant inhibition of precipitation in the concentrations used.

The greater effectiveness of ε-N-(α-D-benzylpenicilloyl)-aminocaproate (IId) over the N-(α-D-benzylpenicilloyl) derivatives of ammonia (IIa), benzylamine (IIf) and *n*-propylamine (IIb) is consistent with the view that the rabbit antibenzylpenicilloyl antibodies are directed also against the lysine side chain (IIi) of the carrier protein. The lesser effectiveness of N-(α-D-benzylpenicilloyl) *n*-

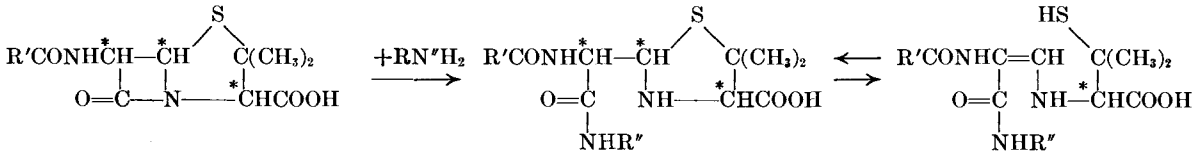
(7) The *in vivo* reaction of benzylpenicillin with protein lysine ε-amino groups appears to proceed through the intermediate, *d*-benzylpenicillenic acid.[3,4] The resulting ε-N-(α-D-benzylpenicilloyl)-lysine antigenic determinant groups are formed as the diastereoisomeric mixture, against which are directed the rabbit antibenzylpenicilloyl antibodies.[5]

(8) K. Landsteiner, *Biochem. Z.*, **98**, 117 (1919); **104**, 280 (1920).

(9) L. Pauling, D. Pressman, D. Campbell, and C. Ikeda, *J. Am. Chem. Soc.*, **64**, 3003 (1942); L. Pauling, D. Pressman, and A. S. Grossberg, *J. Am. Chem. Soc.*, **66**, 784 (1944).

TABLE I

N-(α-D)-PENICILLOYL) AMINES, BENZYLAMINE SALTS, α-DIASTEREOISOMERS



II a b c d e f g h i

R' $C_6H_5CH_2$— ; g: 2,6-dimethoxyphenyl ; h: $CH_2{=}CH{-}CH_2{-}S{-}CH_2$— ; i: $C_6H_5CH_2$—

R" —H ; $-(CH_2)_2CH_3$; $-(CH_2)_4CH_3$; $-(CH_2)_5COOH$; $-(CH_2)_7CH_3$; $-CH_2C_6H_5$; $-(CH_2)_2CH_3$; $-(CH_2)_5COOH$; i: $-(CH_2)_4-CH(CO-)(NH-)$ (Protein Residue)

	R'	R"	M.p., °C.[b]	$[\alpha]^{25}D$	$[\alpha]^{25}D$ of diastereoisomeric mixture[d]	ε at λ_{max} 285 mμ after reaction with PCMB[h]	IP_{50},[k] moles per liter
IIa	$C_6H_5CH_2$—	H—	124–126	+103	+47.5[e,f]	22,850	3.86×10^{-5}
IIb	$C_6H_5CH_2$—	$CH_3(CH_2)_2$—	139–140	+96.6	+50.8[e,f]	23,350	2.85×10^{-5}
IIc	$C_6H_5CH_2$—	$CH_3(CH_2)_4$—	143–144	+91.1	+48.0[f]	23,400	3.41×10^{-5}
IId	$C_6H_5CH_2$—	HOOC—$(CH_2)_5$—	111–112	+70.0	+36.1[e,f]	23,800	1.63×10^{-5}
IIe	$C_6H_5CH_2$—	$CH_3(CH_2)_7$—	125.5–126 (U)	*g*	*g*	23,100	1.36×10^{-4}
IIf	$C_6H_5CH_2$—	$C_6H_5CH_2$—	134–135.5 (U)[c]	*g*	*g*	25,400	3.53×10^{-5}
IIg	2,6-dimethoxyphenyl	$CH_3(CH_2)_2$—	160–160.5 (U)	+84.5	+14.6[f]	22,500	9.27×10^{-3}
				+65.7	+35.8[f]	*i*	7.36×10^{-4}
IIh	$CH_2{=}CHCH_2SCH_2$—	$HOOC(CH_2)_5$	95–96 (U)				

TABLE I (*Continued*)

[a] Performed by Schwartzkopf Laboratories, Middle Village, N. Y. [b] Melting points are capillary and corrected, except for (U) which are uncorrected. [c] Reported m.p. 135–136.[6a] [d] 0.5 to 1.0% in 0.15 *M* sodium chloride solution. [e] Prepared from α-diastereoisomer by cupric ion method (see Experimental). [f] Prepared from α-diastereoisomer by reflux method (see Experimental). [g] Not done because of low water solubility of these compounds. [h] An aqueous solution of the penicilloyl amine was treated with a 15-fold molar excess of *p*-chloromercuribenzoate to form the λ max. 285 mμ absorbing substances, penamaldoyl amines (see Text). The absorption of penicilloyl amines at 285 mμ was negligibly small except for N-(α-D-dimethoxyphenylpenicilloyl) *n*-propylamine, which exhibited an absorption peak at 280 mμ with ε 1980 at 285 mμ. [i] Not reported because of instability of absorption peak. [k] Concentration of penicilloyl amine (diastereoisomeric mixture) required to achieve 50% inhibition of the specific precipitation of rabbit antipenicillin antibody by a benzylpenicilloyl-human *gamma*-globulin antigen.

		Calcd.			Analyses, %[a] Found		
Col.	Formula	C	H	N	C	H	N
(IIa)	$C_{23}H_{30}N_4O_4S \cdot 0.5H_2O$	59.09	6.68	11.98	59.14	6.71	11.42
(IIb)	$C_{26}H_{36}N_4O_4S \cdot 0.5H_2O$	61.30	7.32	10.99	61.93	7.33	10.44
(IIc)	$C_{28}H_{40}N_4O_4S \cdot 0.5H_2O$	62.54	7.68	10.41	62.77	7.60	10.41
(IId)	$C_{36}H_{49}N_5O_6S \cdot H_2O$	61.94	7.37	10.00	62.12	7.61	10.00
(IIe)	$C_{31}H_{47}N_4O_4S \cdot 0.5H_2O$	64.10	8.33	9.64	63.77	8.33	9.68
(IIg)	$C_{27}H_{38}N_4O_6S$	59.34	7.01	10.24	59.81	7.33	9.81
(IIh)	$C_{33}H_{49}N_5O_6S_2 \cdot 0.5H_2O$	58.10	7.35	10.24	58.05	7.80	10.38

TABLE II: SPECIFIC INHIBITION OF PASSIVE CUTANEOUS ANAPHYLAXIS BY PENICILLOYL AMINES[a]

	Rabbit anti-penicillin serum dilutions in normal saline					Rabbit anti-ovalbumin serum 0.04 μg. antibody nitrogen
Penicilloyl amine injected (10 μmoles)[b]	1/250	1/500	1/1000	1/2000	1/3000	
None	18[c]	14	8	4.5	Trace	13
ε-N-(α-D-Benzylpenicilloyl)aminocaproic acid, IId	Trace	0	0	0	0	14
N-(α-D-Benzylpenicilloyl)-*n*-propylamine, IIb	13	7	6	0	0	13
N-(α-D-Benzylpenicilloyl) amide, IIa	13	11	7	0	0	15
D-α-Benzylpenicilloic acid (monosodium salt)	15	12	7	0	0	15
N-(α-D-Dimethoxyphenylpenicilloyl)-*n*-propylamine, IIg	17	14	10	Trace	Trace	17

FOOTNOTES TO TABLE II

[a] Albino guinea pigs 250–300 g. were used. The diluted serum was injected into the skin. After 4 hr. (latent period), the hapten was injected intravenously followed in 5 min. by the antigenic mixture (250 μg. of benzylpenicilloyl-human *gamma*-globulin, 75 μ. of ovalbumin, 0.5 ml. of 1% Evans blue; total volume, 1 ml.) Guinea pigs were killed 15 min. after injection of antigens. Reactions appeared in 3 min. and were at a maximum within 7 min. after injection of antigen. [b] Hydrated benzylamine salts, diastereoisomeric mixtures prepared by refluxing a solution of the α-diastereoisomer for 10 min. (see Experimental). [c] Diameter of bluing reaction in mm. Each value is the average of the reaction diameters in 3 animals.

octylamine (IIe) and N-(α-D-benzylpenicilloyl) *n*-amylamine (IIc) is due to their stronger nonspecific binding to serum albumin.[10] The considerably lesser effectiveness of N-(α-D-dimethoxyphenylpenicilloyl) *n*-propylamine (IIg) and ε-N-(α-D-allylmercaptomethylpenicilloyl)-aminocaproate (IIh) in inhibiting precipitation suggests that the rabbit antibenzylpenicilloyl antibodies are directed against the entire ε-N-(α-D-benzylpenicilloyl)-lysine determinant. Consistent with this possibility are the observations that dimethoxyphenylpenicilloyl-human *gamma*-globulin and allylmercaptomethylhuman *gamma*-globulin antigenic conjugates could precipitate only a small fraction of the rabbit antibenzylpenicilloyl antibodies.[11] These observations suggest that it may be possible to prepare biosynthetic penicillins[12] with side chains sufficiently different in structure from the benzyl group, so that their allergic cross-reactivity with benzylpenicillin may be very slight, or occur not at all.

Hapten inhibition of passive cutaneous anaphylaxis (PCA) reactions given by the rabbit antibenzylpenicilloyl antibodies and the benzylpenicilloyl-human *gamma*-globulin antigen was carried out in guinea pigs by methods originally described by Ovary and Karush.[13] Table II shows that the abilities of the mutarotated penicilloyl amines to specifically inhibit the PCA reaction generally paralleled their abilities to inhibit precipitation *in vitro*. Prior injection of 10 μmoles (7 mg.) of the diastereoisomeric di-benzylammonium ε-N-(α-D-benzylpenicilloyl)-aminocaproate salt caused complete inhibition of the PCA reaction, whereas prior injection of equimolar quantities of the other benzylpenicilloyl haptens caused incomplete inhibition. Complete inhibition of PCA reaction was achieved with the other benzylpenicilloyl haptens by increasing the quantities injected, *e.g.*,

(10) B. B. Levine, to be published.
(11) B. B. Levine, *Fed. Proc.*, **21**, 32 (1962).
(12) D. C. Hobbs and A. R. English, *J. Med. Pharm. Chem.*, **4**, 207 (1961).
(13) Z. Ovary and F. Karush, *J. Immunol.*, **84**, 409 (1960).

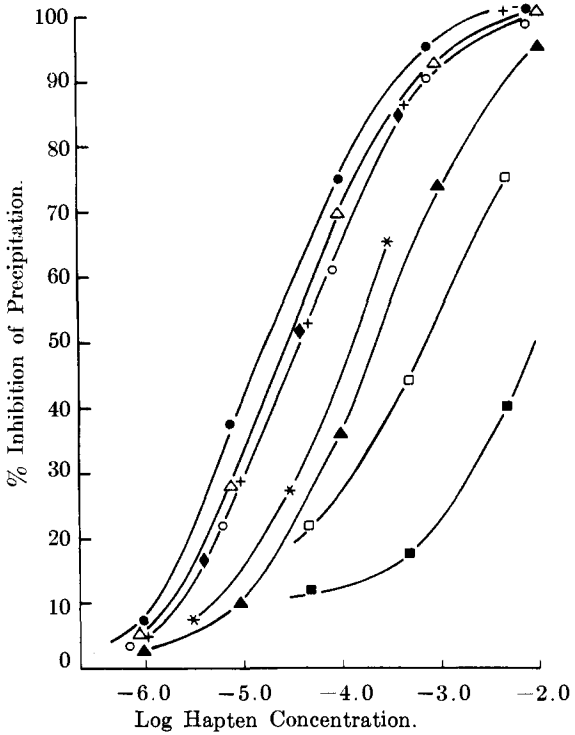


Fig. 1.—Inhibition of precipitation of rabbit anti-penicillin serum by benzylpenicilloyl-human gamma globulin with penicilloyl-amine univalent hapten:

● IId △ IIb ◆ IIf ○ IIa + IIc * IIe □ IIh ■ IIg ▲ —Monosodium D-α-benzylpenicilloate

for monosodium benzylpenicilloate 260 μmoles (100 mg.). Prior injection of 10 μmoles of the dimethoxyphenylpenicilloyl hapten (IIg) caused no detectable inhibition of PCA. These univalent haptens were incapable of provoking PCA reaction. Inhibition of PCA reaction was specific as benzylamine in the quantities used did not inhibit PCA reaction, nor did the penicilloyl amines inhibit PCA given by the ovalbumin-rabbit-antiovalbumin system (Table II). No overt signs of toxicity were seen in the guinea pigs injected with penicilloyl amines.

From these data, and considering that high percentages of patients with recent allergic reactions to penicillin show allergic skin reactivity to benzylpenicilloyl conjugates,[10] it is possible that penicilloyl amines may prove to be useful as therapeutic agents to prevent and treat

antibody-dependent allergic reactions to penicillin in man. The general method of hapten inhibition, which has been a fruitful tool in experimental immunochemistry, may thus find application also in clinical medicine. Studies on human beings are in progress.

Experimental

Materials and Methods.—Crystalline sodium benzylpenicillin was obtained from Pfizer Laboratories, Brooklyn, N. Y., sodium dimethoxyphenylpenicillin (Staphcillin) from Bristol Laboratories, Syracuse, N.Y., and sodium allylmercaptomethyl penicillin (Cer-O-Cillin) from Upjohn Laboratories, Kalamazoo, Michigan. *n*-Propylamine and *n*-amylamine were obtained from Pennsalt Chemicals Corporation, Philadelphia, Pa. *p*-Chloromercuribenzoic acid was obtained from Amend Drug Company, New York, N.Y. For use in the penamaldate analytic method, it was dissolved in the minimum amount of 0.1 *N* sodium hydroxide, made up to volume with 0.05 *M* pH 9.2 carbonate buffer, and analyzed by the method of Boyer.[14] This reagent was stored at room temperature and was stable for several months. The 0.05 *M* pH 9.2 carbonate buffer was stored at 4° in a tightly stoppered polyethylene bottle to prevent loss of carbon dioxide. It was stable for several months. Monosodium D-α-benzylpenicilloate was prepared as described previously, m.p. 154–155° (micro.).[3] Other chemicals were of reagent grade.

Ultraviolet absorption spectra were taken with a Zeiss Model PMQ II spectrophotometer using 1 cm. matched quartz cuvettes. Optical rotations were taken with a Schmidt and Haensch polarimeter with a 2-dm. cell.

Preparation of the α-Diastereoisomers of N-(α-D-Penicilloyl) Amines and Amino Acids.—To a solution of 14 mmoles of the penicillin (5.0 g. sodium benzylpenicillin, 4.8 g. of sodium allylmercaptomethylpenicillin, or 5.6 g. sodium dimethoxyphenylpenicillin) in 100 ml. of H_2O was added 1.2 *M* equivalents of the amine and the reaction solution was stirred for 20 min. at room temperature. For the reaction of penicillin with ε-aminocaproic acid, 1 *M* sodium hydroxide was added to the reaction mixture to maintain the pH between 11.5 and 12.0. In all cases, the reactions were complete within 10 min. This was indicated by a rapid fall of optical rotation to reach a stable value within 10 min. For the reaction with ε-aminocaproic acid, the pH was maintained at 11.7 without further addition of sodium hydroxide after 10 min. had elapsed. The reaction solution was brought to pH 1.5 with dil. phosphoric acid (1:4 H_2O by volume), and the precipitated penicilloyl amine was gathered by filtration, washed with cold water and dissolved in the 1-butanol–ether (12:88 by volume) extract of the reaction solution. The organic solution was dried over anhydrous sodium sulfate, benzylamine (1.5 *M* equivalents) was added, and the penicilloyl amines were crystallized from the reaction mixture as white needles. The product was generally recrystallized from 95% ethanol-ether. Yields ranged between 50–80%. The benzylamine salts could be converted quantitatively to sodium salts by passage of a solution of the benzylamine salt through a Dowex 50 (Na^+ cycle) column at pH 7. The impure sodium salts of the penicilloyl amines could be isolated from the reaction mixture in 90% yields by lyophilizing the reaction mixture, extracting the light yellow powder with warm absolute ethanol, and precipitating the product by addition of

(14) P. D. Boyer, *J. Am. Chem. Soc.*, **76**, 4531 (1954).

two volumes of dry ether to the ethanol solution. Analyses of the resulting amorphous white powder by the penamaldate method described below showed a penicilloyl amine assay of 85 to 95%.

Preparation of the Diastereoisomeric Mixtures of the Penicilloyl Amines by Mutarotation of the α-Diastereoisomers.—Two methods were used. The mutarotation of ε-N-(α-D-benzylpenicilloyl)-aminocaproic acid is given as an example. (1) A $2.00 \times 10^{-2}M$ solution of the α-diastereoisomer, dibenzylamine salt dissolved in water or in 0.15 M aqueous sodium chloride was adjusted to pH 5.0, cupric sulfate was added to a final concentration of $1.00 \times 10^{-4}M$, and the solution was incubated at 37° under nitrogen. The optical rotation fell from an initial value of $\alpha^{25}D$ +1.94° to reach a stable value of $\alpha^{25}D$ +1.00° in 2.5 hr. At this time the mutarotated solution showed a penamaldoyl amine (III) concentration of 8.0×10^{-5} M (by ultraviolet spectrophotometry assuming ϵ 23,800 at λ_{max}. 285 mμ) and a penicilloyl amine concentration of $2.00 \times 10^{-2}M$ by penamaldate analyses (see below). Thus aside from the formation of 0.4% penamaldoyl amine by rearrangement, there was no other detectable degradation of penicilloyl amine during this partial racemization. Under the same conditions but at pH 7.5, mutarotation was complete within 5 hr. At pH 7.5 in the absence of cupric ion, more than 100 hr. was required for mutarotation to go to completion. When mutarotation was carried out at pH 7.5, in the presence of cupric ion and under air, the resulting solution contained the penamaldoyl amine at a concentration of 8 to 15% of the original penicilloyl amine concentration. Nitroprusside tests done on these solutions were negative which indicates that the penamaldoyl amines may be present mainly as the oxidized (disulfide) form. Mutarotation of *n*-amylamine and *n*-octylamine derivatives could not be accomplished by this method as addition of cupric ion to the penicilloyl amine solution caused precipitation of a copper complex.

(2) A $2.00 \times 10^{-2}M$ solution of the ε-N-(α-D-benzylpenicilloyl)-aminocaproic acid, dibenzylamine salt in 0.15 M aqueous sodium chloride solution (pH 5.5) was refluxed for 10 min. under air. The optical rotation fell to + 0.98°. The mutarotated solution contained penamaldoyl amine at a concentration of 1.5% of the original penicilloyl amine concentration. It was necessary to use dilute solutions ($5 \times 10^{-4}M$) of the *n*-octylamine derivative because of its limited solubility.

Quantitative Assay of Penicilloyl Amines (Penamaldate Method).—To 5 ml. of the penicilloyl amine at a concentration of approximately $2 \times 10^{-5}M$ in 0.05 M carbonate buffer, pH 9.2, is added 0.10 ml. of a $1.50 \times 10^{-2}M$ stock solution of *p*-chloromercuribenzoate (PCMB) (see Materials). The optical density at 285 mμ of this solution is taken between 5 and 15 min. after mixing. This optical density is corrected for the contribution of 3.00×10^{-4} M PCMB (subtract 0.060 optical density units), and this value is corrected for dilution by multiplication by 1.02. The penicilloyl amine concentration is calculated by dividing the corrected optical density by the molar extinction coefficient (Table I). At pH 8.5 to 10.0 and using 4–50 moles of PCMB per mole of penicilloyl amine, the λ 285 mμ peaks forms immediately and thereafter the absorbancy at λ 285 mμ falls slowly. The optical density taken at 15 min. is 2 to 3% lower than the optical density at 5 min. except in the case of ε-N-(α-D-allylmercaptomethylpenicilloyl)-aminocaproate where the absorbancy at λ 285 mμ decreases more rapidly. The maximum error incurred because of the slight instability of the λ 285 mμ peak is thus less than 1.5%. The standard molar extinction coefficients for the peni-

cilloyl amines are listed in Table I and were calculated from the average of the optical densities taken at 5 and at 15 min., which were corrected for the contribution of PCMB as described above. Under these conditions, the corrected optical density at λ 285 mμ and penicilloyl amine concentration followed Beer's law.

Immunological Studies.—Rabbits were immunized by injection of a preincubated mixture of potassium benzylpenicillin and normal rabbit serum; the mixture was emulsified in complete Freund's adjuvant. The sera of 6 animals were pooled. The pooled antiserum was found to contain 380 μg. of antibenzylpenicilloyl antibody protein per ml. as determined by quantitative precipitation reaction[15] (0.50 ml. aliquots of serum in total volume of 2.00 ml. were analyzed). The antigenic benzylpenicilloyl-human *gamma*-globulin conjugate was prepared by the reaction of D-benzylpenicillenic acid[4] with Human Fraction II (American Red Cross) and several purification steps. This antigen (HGG-BPO-CSH-A) contained an average of 23 benzylpenicilloyl groups (diastereoisomeric mixture) per molecule bound predominantly to lysine ϵ-NH_2 groups. The details of immunization and preparation of the antigen have been given previously.[5] Hapten inhibition experiments were set up at the equivalence point. Duplicate tubes contained 0.50 ml. of antisera, 72 μg. of antigen, and hapten in various quantities (total volume 2.00 ml.; diluent buffered saline, pH 7.3). The tubes were incubated 1 hr. at 37°, and 48 hr. at 4°, which was sufficient to give maximum precipitation. Precipitates were washed three times with ice-cold saline and analyzed by the Folin method as described previously.[5] The uninhibited tube yielded a precipitate of 260 μg. of protein; serum and antigen controls yielded approximately 5 μg. of protein. Values plotted in Fig. 1 are averages of duplicate analyses; mean deviation, ± 3%. Techniques of inhibition of PCA reactions with haptens are given in the legend of Table II, and have been described previously.[5,13]

Acknowledgment.—The competent technical assistance of Miss Arline Sobel is gratefully acknowledged. We wish to thank Dr. Norton Nelson for permission to carry out some of these experiments in the Laboratories of the Department of Industrial Medicine.

(15) E. A. Kabat and M. F. Mayer, "Experimental Immunochemistry," C. C Thomas, Springfield, Ill., 2nd ed., 1961, Chap. 2.