pounds (VI, VII, and X) were also virustatic. Compounds V and VI were virucidal against vaccinia in tissue culture; VI, X, and XIV showed activity against the hepatitis viral strain in mice. These results support the soundness of the assumption on which this research was planned, that ketoaldehydes derived from steroidal components would be antiviral.

Experimental[5]

All α-ketoaldehydes discussed in this note have been described in the literature. We prepared them in good yield by oxidation of the corresponding ketols with oxygen in aqueous methanol solution. Chemical and physical characteristics (especially infrared frequencies and rotatory indices) were in accord with literature data.

Preparation of Schiff's Bases and N,N-Diacetals.—A mixture of the ketoaldehyde (1 mmole) and the respective primary amine (1–2 mmoles) in 10 ml. of ethanol was stirred at 20° for 24 hr., and the solution was concentrated until crystallization took place.

(5) All melting points are corrected.

The Preparation of Penicilloyl–Polylysines, Skin Test Reagents for the Clinical Evaluation of Penicillin Hypersensitivity

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The benzylpenicilloyl (BPO) group has been demonstrated to be the major haptenic antigenic determinant of benzylpenicillin (PG) hypersensitivity.[1-3] Multivalent benzylpenicilloyl–polylysine (BPO–PLL) conjugates have been found to be effective elicitors of allergic skin reactions of the wheal-and-flare reactions in a significant percentage of patients with histories of allergy to PG.[2,3] These materials are accordingly promising skin test reagents for the clinical evaluation of penicillin hypersensitivity. Penicilloyl–PLL conjugates have been prepared previously by reaction of penicillenic acids with polylysine.[1-4] This procedure is tedious and results in conjugates contaminated with penicillenic acid groupings and with other impurities. This paper reports a new and simple method for the preparation of comparatively clean succinoylated multivalent penicilloyl–PLL(S) conjugates of different extents of conjugation directly from penicillins. This method is based on the known reaction of penicillins with aliphatic amines at high pH to form penicilloylamines.[5,6] Parker and Thiel have recently published a method of preparation of maximally coupled, unsuccinoylated penicilloyl–PLL conjugates based on this reaction.[7]

Table I shows the relation between mole ratios of reactants and the extents of conjugation of penicilloyl–PLL(S) conjugates which were prepared from PLL preparations of four different degrees of polymerization and from four different penicillins. Over 100 preparations have been made with similar results. Under the same conditions, penicillins were made to react with poly-D-lysine to form succinoylated multivalent penicilloyl–poly-D-lysine conjugates. This preparative method thus appears to be a general one.

The extents of conjugation listed in Table I are 7% too low, as 7% of the penicilloyl groups undergo N^4-thiazolidine acylation during the succinoylation reaction. N^4-Acylated penicilloyl residues do not undergo the penamaldate reaction[5,6] which is the basis of the penicilloyl assay. A maximum of only 60% of the NH_2 groups of PLL could be coupled with penicilloyl groups, probably because of steric interference from the bulky penicilloyl groups. Succinoylation, under the conditions given in the Experimental section, coupled at least 97% of the NH_2 groups, as determined by formol titrations.[8] The conjugate solutions were found to be free from unreacted penicillins by bioassay[9] and free from benzylpenicilloic acid by arsenomolybdate reduction.[10] The ultraviolet absorption spectrum of benzylpenicilloyl–PLL(S) conjugates showed absorption peaks at 278 mμ which corresponds to the presence, in the conjugates, of penamaldoyl groups[5] formed by rearrangement of penicilloyl groups.[5] The optical densities at 280 mμ of some typical conjugate solutions indicate that 1 to 3% of the penicilloyl groups contained in the conjugates exist as the tautomeric penamaldate form. The absorption spectra show also superimposed peaks at λ 258 and 264 mμ corresponding to the benzyl side chain,[5] and another peak at λ 268 mμ which may indicate trace quantities of penaldate groups.[5] There were no detectible benzylpenicillenic acid disulfide chromophoric groupings detectible in the conjugate solutions as evidenced by the absence of absorption maxima in the 310–340 mμ region.[5]

Optical rotations of the conjugate solutions corrected for the contribution of succinoylated PLL yielded $[\alpha]^{25}D$ +0.96° for $1 \times 10^{-2}M$ benzylpenicilloyl contained in a typical benzylpenicilloyl–PLL(S) conjugate, a value in excellent agreement with the molar specific rotations of α-diastereoisomeric crystalline univalent benzylpenicilloylamines.[6] This finding indicates that the penicilloyl groups contained in the conjugates prepared by the method given here are entirely, or predominantly, α-diastereoisomers, the expected diastereoisomeric product of the reaction of penicillins with amines at high pH.[5,6] In contrast, penicilloyl–polylysines prepared from penicillenic acids are diastereoisomeric mixtures.[1] Optical rotations of benzylpenicilloyl–PLL(S) solutions (BPO_{80}–PLL_{102}(S) and BPO_{60}–PLL_{286}(S)) taken at decreasing pH showed

(1) B. B. Levine and Z. Ovary, *J. Exptl. Med.*, **114**, 875 (1961).
(2) C. W. Parker, J. Shapiro, M. Kern, and H. N. Eisen, *ibid.*, **115**, 821 (1962).
(3) B. B. Levine, and V. H. Price, *Immunology*, in press.
(4) B. B. Levine, *J. Exptl. Med.*, **117**, 161 (1963).
(5) "Chemistry of Penicillin," H. T. Clarke, J. R. Johnson, and R. Robinson, Ed., Princeton University Press, Princeton, N. J., 1949.
(6) B. B. Levine, *J. Med. Pharm. Chem.*, **5**, 1025 (1962).
(7) C. W. Parker and J. A. Thiel, *J. Lab. Clin. Med.*, **62**, 482 (1963).
(8) A. W. Kenchington in "A Laboratory Manual of Analytic Methods in Protein Chemistry," P. Alexander and R. J. Block, Ed., Pergamon Press, New York, N. Y., 1960, p. 353.
(9) D. G. Grove and W. H. Randall, "Assay Methods of Antibiotics," Medical Encyclopedia, Inc., New York, N. Y., 1955, pp. 14–16.
(10) S. C. Pan, *Anal. Chem.*, **26**, 1438 (1954).

TABLE I

RATIO OF NH_2 GROUPS IN POLYLYSINES CONVERTED TO PENICILLOYLAMINES BY REACTION WITH PENICILLINS[a]

Mole ratio of reactants penicillin–ε-NH_2 group	Benzylpenicillin plus PLL_{286}	Benzylpenicillin plus PLL_{402}	Benzylpenicillin plus PLL_{525}	Benzylpenicillin plus PLL_{999}	Dimethoxyphenylpenicillin plus PLL_{402}	Allylmercaptomethylpenicillin plus PLL_{402}	Oxacillin[b] plus PLL_{402}
0.3	...	0.08; 0.09[c]	...	...	0.08	...	...
0.7	0.25; 0.21	0.18; 0.23; 0.20	0.18; 0.21	0.23	0.19	...	...
1.0	...	0.23; 0.26	0.21; 0.29	...	0.22	0.22	0.26
3.0	...	0.35	0.37	...	0.35	...	...
10.0	...	0.50; 0.55	0.48	...	0.53	...	...

[a] Penicilloyl–polylysines were succinoylated (see text). [b] 5-Methyl-3-phenyl-5-isoxazolylpenicillin. Molar extinction coefficient of penamaldoyl derivative assumed to be 23,000 at λ 285 mμ. [c] Each value represents an individual preparation.

constant $[\alpha]^{25}D$ values between pH 10 and 6, and a sharp increase of $[\alpha]^{25}D$ starting at pH 5.5. These observations indicate that the conjugate molecules exist, at least in part, as randomly coiled chains at neutral pH, and assume α-helical configuration at acid pH.[11]

The α-diastereoisomeric penicilloyl–PLL(S) conjugates prepared from penicillins are at least as effective as are the diastereoisomeric penicilloyl–PLL(S) prepared from penicillenic acids in eliciting immediate allergic reactions in the skins of human beings hypersensitive to penicillin and guinea pigs passively sensitized with rabbit antibenzylpenicillin sera. Some typical data are given in Table II. Allergic skin reactions elicited by the BPO–PLL(S) conjugates could be completely and specifically inhibited by the univalent hapten, ε-BPO–aminocaproate,[1,9] confirming the BPO haptenic specificity of these allergic reactions. Fully succinoylated penicilloyl conjugates of poly-L-lysine and of poly-D-lysine were found to be nonantigenic in guinea pigs[12,13] and, accordingly, may be nonantigenic in man. Further studies on the usefulness of these conjugates as clinical reagents to detect penicillin hypersensitivity in man are in progress.

TABLE II

COMPARATIVE EFFECTIVENESS TO ELICIT IMMEDIATE ALLERGIC SKIN REACTIONS OF PENICILLOYL–POLYLYSINES PREPARED FROM PENICILLIN AND FROM BENZYLPENICILLENIC ACID

Wheal-and-Flare Reactions of Patients Hypersensitive to Benzylpenicillin[b]

Patient	Concentration of α-BPO_{58}–$PLL_{402}(S)$[a] 1γ/ml.	Concentration of α-BPO_{58}–$PLL_{402}(S)$[a] 10γ/ml.	Concentration of DM-BPO_{53}–$PLL_{402}(S)$[a] 1γ/ml.	Concentration of DM-BPO_{53}–$PLL_{402}(S)$[a] 10γ/ml.
SL	7;15[c]	11;30[c]	8;15[c]	11;30[c]
JB	9;30	10;40	7;30	10;40
JN	9;35	10;35	8;35	10;35
WS	7;20	8;20	6;20	7;30

Cutaneous Anaphylactic Reactions of Guinea Pigs Passively Sensitized with Rabbit Antibenzylpenicillin Sera[c]

Guinea pig no.	Concentration of α-BPO_{58}–$PLL_{402}(S)$[a] 0.1γ/ml.	Concentration of α-BPO_{58}–$PLL_{402}(S)$[a] 1.0γ/ml.	Concentration of DM-BPO_{53}–$PLL_{402}(S)$[a] 0.1γ/ml.	Concentration of DM-BPO_{53}–$PLL_{402}(S)$[a] 1.0γ/ml.
1	10S	11S	10S	11S
2	11M	11MS	10M	10M
3	12S	15S	12MS	14S

[a] α-BPO_{58}–$PLL_{402}(S)$ contains α-diastereoisomeric benzylpenicilloyl BPO groups. DM-BPO_{53}–$PLL_{402}(S)$, prepared from benzylpenicillenic acid contains a diastereoisomeric mixture of BPO groups. Subscripts refer to average numbers of residues per mole of conjugate. [b] Average diameters (mm.) of wheal;flare. Duplicate tests were done; mean deviation for wheal, ±1.0 mm. [c] Average diameters (mm.) and color intensities of reactions in 3 individual guinea pigs sensitized with a given rabbit antipenicillin serum; S = strong, MS = moderate strong, M = moderate.

Experimental

Potassium benzylpenicillin, sodium dimethoxyphenylpenicillin (Staphcillin®), and sodium 5-methyl-3-phenyl-4-isoxazolylpenicillin (Prostaphlin®) were generously supplied by Bristol Laboratories, Syracuse, New York. Potassium allylmercaptomethylpenicillin (Cer-O-Cillin®) was a gift from the Upjohn Laboratories, Kalamazoo, Michigan. PLL·HBr was purchased from Pilot Laboratories, Watertown, Massachusetts. Four preparations with average degrees of polymerization of 286, 402, 525, and 999, based on intrinsic viscosities (manufacturers' analyses) were used. Penicillins were allowed to react with polylysine (PLL) in aqueous solution at room temperature (22–27°) in a pH-stat for 90 min. with the pH maintained at 11.5 by additions of 1 *M* NaOH. The reaction solutions were then made to react 3 times with 3 molar equiv. (with respect to ε-amino groups) of succinic anhydride, once at pH 11.5 and twice at pH 9.5 to 10.0 in order to succinoylate the free amino groups of the penicilloyl-PLL conjugates. The succinoylated conjugates (penicilloyl-PLL(S)) were freed from low molecular weight impurities by *prolonged dialysis against amberlite* IRA-400 *ion-exchange* resin suspended in 0.002 *M* Tris buffer (pH 8.5)[1] with a final dialysis against 0.003 *M* phosphate buffer (pH 8.2). The conjugates were stored in solution at 4° without preservative where they were stable over a 6-month period of observation. Conjugate solutions were analysed for PLL base by duplicate micro-Kjeldahl[14] analyses corrected for the nitrogen contribution of penicilloyl groups, and analyzed for penicilloyl concentrations by the penamaldate method.[1,6] DM-BPO_{53}-$PLL_{402}(S)$ was prepared by treating PLL_{402} with benzylpenicillenic acid.[1,4]

Patients with recent late urticarial allergic reactions to benzylpenicillin were skin tested. Test doses (0.003 ml.) were injected intradermally in the deltoid areas. Wheal-and-flare reactions were measured at 15 min. Duplicate tests were done (mean deviation for wheal ±1.0 mm.). Reagent controls in nonsensitized patients and diluent controls in test patients were negative (<2 mm. wheal).

Rabbit antibenzylpenicillin sera were prepared as reported previously.[1] Albino guinea pigs weighing 275–325 g. were sensitized by intravenous injection of rabbit antiserum containing 250 γ of antibenzylpenicilloyl antibody protein. After a latent period of 48 hr., animals were injected i.v. with 0.5 ml. of 1% Evans blue dye, and challenged by intradermal injections of 0.1 ml. of the conjugate solutions. Positive reactions (circular blue spots) are at their maximal intensities in 15 min. Reagent controls in nonsensitized animals and diluent controls in sensitized animals were negative (2-mm. streaks).

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(11) P. Doty, K. Imahori, and E. Klemperer, *Proc. Natl. Acad. Sci. U. S.*, **44**, 424 (1958).

(12) B. B. Levine, *Proc. Soc. Exptl. Biol. Med.*, in press.

(13) B. B. Levine, *Nature*, **202**, 1008 (1964).

(14) E. A. Kabat and M. F. Mayer, "Experimental Immunochemistry," 2nd Ed., Charles C Thomas, Springfield, Ill., 1961, p. 476.