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Note

Formation of penicillin polymers and determination of molecular weight

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The impurities in penicillins such as proteins and polymers have been investigated[1–3] and the chemical aspects of penicillins have been studied[4–7]. Penicillins form polymers by cleavage of the β-lactam in aqueous solutions at room temperature, and these polymers are considered to be one of the causes of penicillin allergy[8–17].

For the study of penicillin polymer formation, Sephadex G-25 chromatography has been widely used. We, however, found strong adsorption of a few penicillins to the gel and the peaks of penicilloic acids formed by cleavage of the β-lactam appeared in the polymer region. These characteristics make the chromatographic system unsuitable for the determination of the molecular weight of penicillin polymers.

Concin *et al.*[18] reported investigations of the chromatographic behaviour of aromatic compounds on alkylated dextran gel; Sephadex LH-60 was used and a chromatographic system was designed for molecular weight determinations.

This paper describes the formation of polymers of ampicillin, amoxicillin, piperacillin and PL-385 (Fig. 1), and the determination of the molecular weights of the polymers by using Sephadex LH-60.

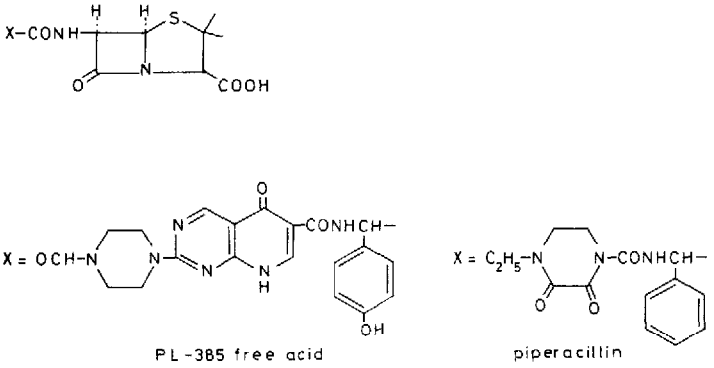

Fig. 1. Structures of PL-385 free acid and piperacillin.

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EXPERIMENTAL

Materials

Ampicillin, amoxicillin and piperacillin were obtained from Dainippon (Osaka, Japan), Dobfar (Milan, Italy) and Toyama Chemical (Tokyo, Japan), respectively. PL-385 was synthesized in our laboratories. Polystyrene Sulfonate 6500 and Polystyrene Sulfonate 1600 were purchased from Pressure Chemical (Pittsburgh, PA, U.S.A.) and Union Carbide (Chicago, IL, U.S.A.), respectively. Sephadex G-25, fine, and Sephadex LH-60 were purchased from Pharmacia (Uppsala, Sweden).

Penicilloic acids were prepared from amoxicillin and PL-385 as follows: 4.2 g of amoxicillin was suspended in 100 ml of ice-cooled water and 1.2 g of sodium hydroxide was dissolved in 50 ml of water and poured into the amoxicillin suspension. After stirring for 1 h in an ice–water bath, the solution was adjusted to pH 6.8 by adding 10% hydrochloric acid dropwise. The reaction mixture was lyophilized and 4.3 g of products were obtained. The penicilloic acid of PL-385 was obtained by a similar procedure. These products were confirmed by their IR and ^{1}H NMR spectra.

Conditions for Sephadex G-25 chromatography

A Shimadzu Model LC-3A liquid chromatograph, equipped with a Model UVD-4 detector (254 nm) and a Model SIL-1A injector (Shimadzu, Kyoto, Japan) was used. The column was a glass tube (1500 × 9 mm I.D.), the flow-rate was 2.0 ml/min and the eluent was prepared by dissolving 10 g of triethylamine and 1 g of sodium chloride in 950 ml of water, adjusting the pH to 9.5 with hydrochloric acid and making up to 1000 ml with water. In order to separate the penicillin polymers, two of the columns joined together were used, and the effluent was fractionated to 4-ml volumes.

Conditions for Sephadex LH-60 chromatography

A Shimadzu Model LC-3A chromatograph was used. The column was a glass tube (250 × 13.6 mm I.D.) and dioxane–water (7:3) was used as the eluent at a flow-rate of 0.1 ml/min.

Polymer formation

The penicillins were dissolved in water to make 0.1 *M* solutions, which were adjusted to pH 8 with 0.1 *N* sodium hydroxide solution and stored at room temperature for 7 days. Small volumes of the solutions were injected into the Sephadex G-25 column for analysis. Part of each solution of the penicillins was dialysed with a cellulose tube at room temperature. After 12 days the solutions of the penicillins were injected into the Sephadex G-25 column for separation and the polymeric substances were separated. Small amounts of the polymeric substances were injected into the Sephadex LH-60 column to determine their molecular weights.

RESULTS

Sephadex G-25 chromatography

The elution patterns are shown in Fig. 2. The distribution coefficients, K_{av}, were calculated according to the equation of Laurent and Killander[19]:

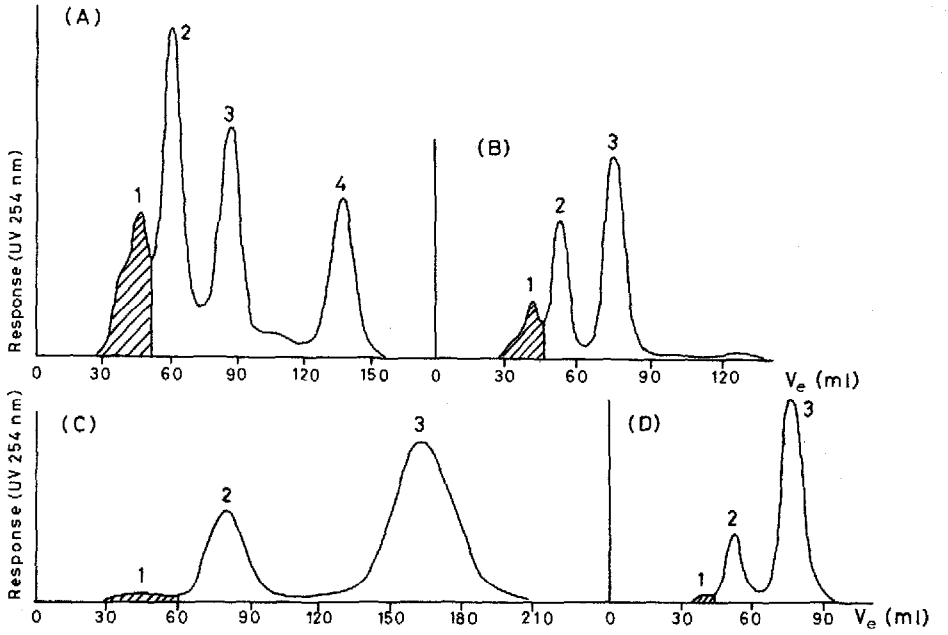


Fig. 2. Chromatograms of 0.1 *M* aqueous penicillin solutions adjusted to pH 8 and stored at room temperature for 7 days. Stationary phase, Sephadex G-25, fine; column, 1500 × 9 mm I.D.; mobile phase, 0.01% sodium chloride and 0.1% triethylamine aqueous solution adjusted to pH 9.5; flow-rate, 1.0 ml/min. (A) Ampicillin; (B) amoxicillin; (C) PL-385; (D) piperacillin. 1 = Polymer; 2 = penicilloic acid; 3 = unchanged penicillin; 4 = unidentified product.

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume of solute, V_t is the bed volume and V_0 is the void volume. In Sephadex G-25 analytical chromatography V_0 was 30 ml, decided by the elution volume of bovine serum albumin, and V_t was 90 ml.

Adsorptivity to Sephadex G-25

For Sephadex G-25 chromatography of penicillin polymers, sodium chloride solutions are often used as the eluent. PL-385 had strong adsorptivity to the gel and using 5% sodium chloride as the eluent K_{av} was 3.7. Although the eluent used in this paper was optimal, the K_{av} value of PL-385 was 1.7.

Gelotte[20] showed that three properties of substances that affect their adsorptivity to Sephadex gels, namely aromaticity, a heterocyclic moiety and basicity; γ-collidine was used as a typical compound. PL-385 has a pyridopyrimidine moiety with all of these properties, which may be the cause of the strong adsorption to the gel.

Chromatographic behaviour of penicilloic acid

Among the peaks shown in Fig. 2, those with the largest K_{av} correspond to penicillin monomers and those with the next largest K_{av}, which usually means a larger molecular weight, correspond to penicilloic acids, contrary to our expectations. This

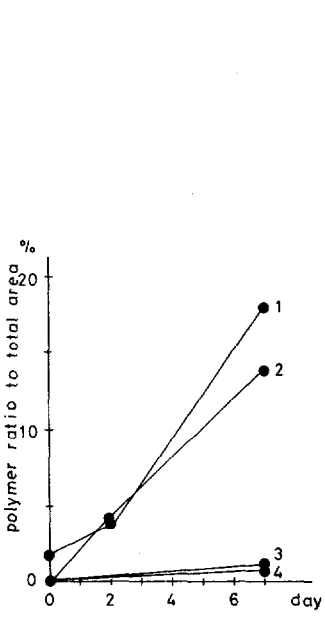
was confirmed by preparing penicilloic acids of PL-385 and amoxicillin. The peaks with the next largest K_{av} disappeared together with penicillin monomers on dialysis with a cellulose tube.

Detection of polymer

The peaks whose K_{av} values were smaller than those of penicilloic acids were assumed to be those of polymers. The time course of the polymerization of penicillins is shown in Fig. 3.

Molecular weight determination

Using Sephadex G-25, penicillins can be compared with respect to susceptibility of polymer formation, but the phenomena observed with PL-385 and penicilloic acids indicate that this gel is not useful for molecular weight determinations. Concin *et al.*[18] used Sephadex LH-60 for the determination of the molecular weight of hydrothermally degraded lignin. This procedure overcomes the undesirable characteristics observed with Sephadex G-25.



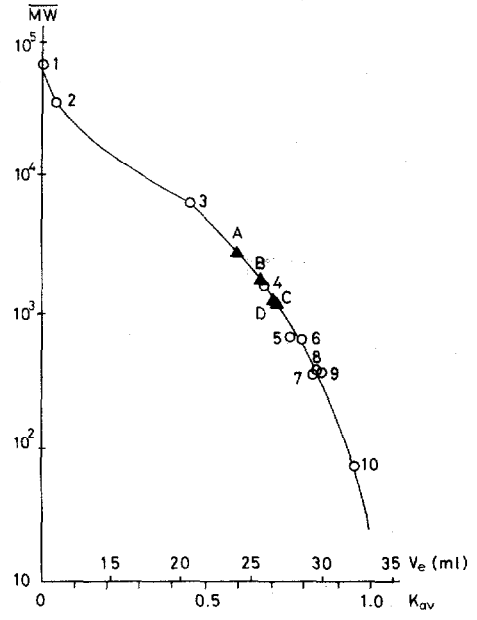


Fig. 3. Time course of polymerization of penicillins in aqueous solutions adjusted to pH 8 and stored at room temperature. 1 = Ampicillin; 2 = amoxicillin; 3 = piperacillin; 4 = PL-385.

Fig. 4. Semi-logarithmic plot of molecular weight *versus* K_{av} values and elution volume (V_e) of polymer standards and penicillin polymers. Stationary phase, Sephadex LH-60; column, 250 × 13.6 mm I.D.; mobile phase, dioxane–water (7:3); flow-rate, 0.1 ml/min. 1 = Bovine serum albumin, MW 68,000; 2 = pepsin, MW 34,500; 3 = polystyrene sulphonate, $\overline{MW}$ 6500; 4 = polystyrene sulphonate, $\overline{MW}$ 1600; 5 = penicilloic acid of PL-385, MW 690; 6 = PL-385, MW 672; 7 = ampicillin, MW 349; 8 = penicilloic acid of amoxicillin, MW 383; 9 = amoxicillin, MW 365; 10 = methyl ethyl ketone, MW 72. A, Polymer of PL-385, $\overline{MW}$ 2500; B, polymer of piperacillin, $\overline{MW}$ 1600; C, polymer of ampicillin, $\overline{MW}$ 1200; D, polymer of amoxicillin, $\overline{MW}$ 1200.

Penicillin solutions stored at room temperature for 12 days were injected into the Sephadex G-25 column for polymer separation. Fractions of polymers were combined and lyophilized and the samples were analysed by Sephadex LH-60 chromatography. V_0 was 10.2 ml, determined by the elution volume of bovine serum albumin, and V_t was 36.2 ml. A calibration graph was obtained by using K_{av} values of Polystyrene Sulfonate 6500, Polystyrene Sulfonate 1600, penicillin monomers and other compounds whose molecular weights were known. The results are shown in Fig. 4. The molecular weights of polymers formed from ampicillin, amoxicillin, piperacillin and PL-385 were calculated to be 1200, 1200, 1600 and 2500, respectively, and these polymers are assumed to be mainly trimers or tetramers. The chromatograms obtained by reversed-phase high-performance liquid chromatography showed that these polymers were mixtures of many products.

DISCUSSION

Because of the low resolution, the chromatographic system using Sephadex LH-60 is not useful for the separation of monomers and polymers formed in penicillin aqueous solutions. No adsorption, however, was shown with PL-385 and the K_{av} values of penicilloic acids were almost identical with those of the original penicillins. Dimer formation of penicilloic acids with a hydrogen or ionic bond in the cleaved β-lactam moiety seems to cause their peaks to appear in the polymer region in Sephadex G-25 chromatography. On the other hand, covalent bonded dimers, for example amide bonded dimers, should have the same K_{av} values as penicilloic acids. From the results of the Sephadex LH-60 chromatography, it is concluded that the products from the polymeric fractions in Sephadex G-25 chromatography, except for the dimer region, are mainly composed of trimers and tetramers.

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