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CHROMATOGRAPHIC SEPARATION AND CHEMICAL ANALYSIS OF POLYMERS FORMED BY PENICILLIN G

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SUMMARY

To elucidate the chemical structures of penicillin polymers that may elicit an allergic reaction, a 25% aqueous solution of penicillin G potassium was kept standing in the dark at room temperature for 14 days and was then separated by gel filtration chromatography on Sephadex G-25. The fractions of K_{av} 0.0-0.3, 0.3-0.55 and 0.55-0.75 were designated fractions A, B and C, respectively.

Chemical and spectral data indicated that fractions A and B had almost similar chemical structures, but differed in molecular weight. They consisted of equimolar phenylacetyl and thiazolidine moieties and showed a C:N:S ratio almost equal to that of penicillin G. Their degrees of polymerization were 10 for A and 3.2 for B.

Comparison of ^1H NMR and IR spectra and thin-layer chromatographic R_F values with those of authentic standards showed that the main components of fraction C were N-formylpenicillamine, benzylpenilloic acid, benzylpenicilloic acid and benzylpenillic acid.

INTRODUCTION

β -Lactam antibiotics, such as penicillins and cephalosporins, are widely used for the treatment of various infectious diseases. However, allergic reactions have been noted in some patients treated with these drugs. The polymers of these antibiotics have been thought to be one of the substances responsible for evoking allergic reactions¹, since polymers in commercial preparations^{2,3} or those prepared by polymerizing the drugs²⁻⁵ have been shown to elicit a passive cutaneous anaphylactic reaction in sensitized animals. To clarify the antigenic determinants relevant to allergic reactions evoked by penicillin G (PcG) administration, the chemical structure of PcG polymers needed to be investigated.

In 1971, Smith and Marshall⁶ proposed structure I (Fig. 1) for the PcG polymers. There have been few studies since on the subject⁷⁻⁹, and I has been cited in

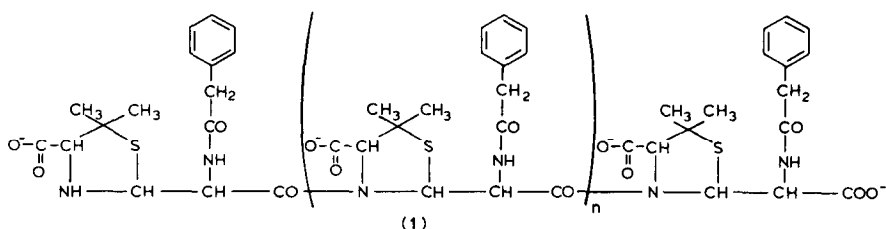


Fig. 1. Chemical structure of penicillin G polymer reported by Smith and Marshall⁶.

many references as the structure. In this study, we tried to isolate polymers formed in an aqueous solution of PcG and studied their chemical structure using various analytical methods.

EXPERIMENTAL

PcG and related compounds

PcG was obtained from Meiji Seika (Tokyo, Japan) and ampicillin (ABPC) from Takeda Chemical Industries (Osaka, Japan). Benzylpenicilloyl- ϵ -aminocaproic acid (BPO-EACA)², dibenzylpenicilloylhexamethylenediamine [(BPO)₂-HMD]¹⁰, benzylpenicilloic acid¹¹, benzylpenilloic acid¹², benzylpenillic acid¹³, N-formylpenicillamine¹⁴, penicillamine disulphide¹⁴ and 2-aminomethyl-5,5-dimethylthiazolidine-4-carboxylic acid (2-AMTC)¹⁵ were prepared by literature methods.

Measurements of ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR) spectra

UV spectra were recorded with a Perkin-Elmer 450B or a Beckman DB-GT spectrophotometer and IR spectra with a Perkin-Elmer 281 spectrophotometer. ¹H NMR spectra were measured with a Varian EM-390 and ¹³C NMR spectra with a Varian XL-100-12 spectrometer.

Thin-layer chromatography (TLC)

TLC was performed using Kieselgel 60F₂₅₄ (Merck) plates and the spots were detected by with palladium(II) chloride, iodine or UV irradiation.

Chemical analysis

Phenylacetyl contents were determined by gas chromatography using a Shimadzu GC-4A gas chromatograph after the polymers had been hydrolysed with 6 *N* hydrochloric acid at 105°C for 24 h in a sealed tube and the liberated phenylacetic acid was converted into its methyl ester with diazomethane. The thiol contents were determined spectrophotometrically¹⁶ using 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; Wako, Osaka, Japan) as a colouring reagent. The non-acylated thiazolidine was measured by a method described elsewhere¹⁷.

Preparation and fractionation of PcG polymers

It was very difficult to obtain a sufficient amount of polymer from the PcG preparation for the analysis, because the polymer present was a very minor one. We

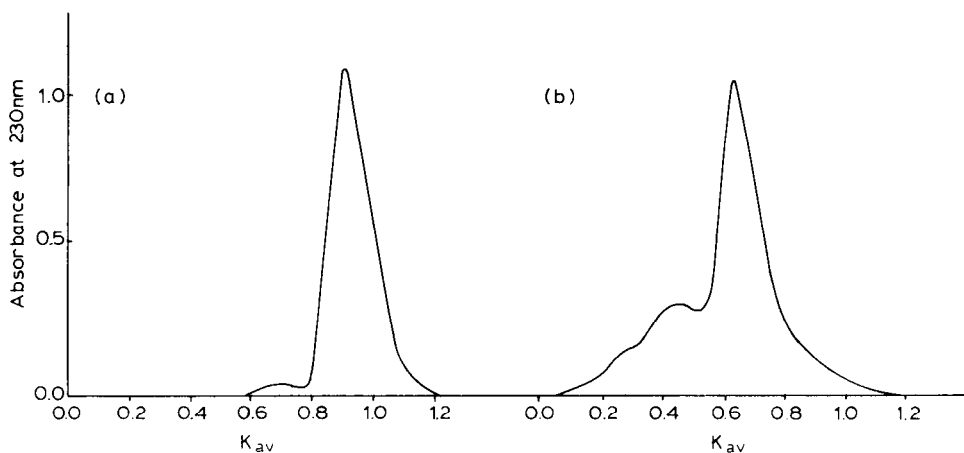


Fig. 2. Gel filtration chromatography of fresh and aged solutions of PcG. Column, Sephadex G-25 fine (100×1.5 cm I.D.); eluent, 0.5% sodium chloride solution; flow-rate, 1 ml/min.

therefore isolated polymers from an aged solution of PcG following the procedure reported by Smith and Marshall⁶.

A solution (25%, w/v) of PcG in sterile water was stored in the dark for 14 days with occasional shaking. The pH of the stored solution was adjusted to about 6.5 by adding sodium hydrogen carbonate powder. The solution (20 ml) was diluted to 30 ml with 0.5% sodium chloride solution, applied to a Sephadex G-25 fine (Pharmacia, Uppsala, Sweden) column (100×5 cm I.D.) and eluted with 0.5% sodium chloride solution. The eluate was fractionated into three parts, with K_{av} 0.0–0.3, 0.3–0.55 and 0.55–0.75. The fraction with K_{av} 0.0–0.3 was rechromatographed under the same conditions. The eluate was applied to a Sephadex G-10 (Pharmacia) column (40×4 cm I.D.), eluted with water and dialysed against water for 16 h using a Spectrapor membrane (molecular cut-off 2000; Spectrum Medical, Los Angeles, CA, U.S.A.). The retentate was lyophilized to give fraction A. The eluate of K_{av} 0.3–0.55 was purified by rechromatography on Sephadex G-25, desalted using a Sephadex G-10 column and then lyophilized to give fraction B. Fraction C was obtained by lyophilization of the eluate of K_{av} 0.55–0.75.

Reversed-phase high-performance liquid chromatography (HPLC)

HPLC was performed with a Varian LC-5000 instrument equipped with a Toyo Soda LS-410 ($5 \mu\text{m}$) column (250×4 mm I.D.). A mixture of acetonitrile and 0.01 M phosphate buffer (pH 7.9) was used as the eluent, the concentration of acetonitrile being increased linearly from 0 to 50% at 1%/min. The flow-rate was 1 ml/min and the absorbance at 254 nm was monitored.

RESULTS AND DISCUSSION

The freshly prepared solution of PcG showed only a peak at K_{av} 0.9 on Sephadex G-25 chromatography (Fig. 2a). After 14 days, the UV-detectable materials were eluted at K_{av} 0 and the peak of PcG at K_{av} 0.9 was no longer detectable (Fig.

TABLE I
ANALYTICAL DATA FOR THE POLYMERS

Parameter	Fraction A	Fraction B	Polymer*
Elemental analysis	C, 45.67; H, 4.67; N, 6.72; S, 7.36. C:H:N:S = 16.6:20.2:2.1:1.0	C, 44.81; H, 4.80; N, 6.61; S, 7.43. C:H:N:S = 16.1:20.1:2.0:1.0	As for Na benzylpenicillin containing 16% water
Degree of polymerization	—	—	3.5
Non-acylated thiazolidine	0.22 μ equiv./mg (1/10 U)	0.71 μ equiv./mg (1/3.2 U)	0.82/3.5 U (1/4.8 U)
Intact β -lactam	Not detected	Not detected	0.07/3.5 U (1/50 U)
Free SH	0.025 μ equiv./mg (1/92 U)	0.06 μ equiv./mg (1/38 U)	0.04/3.5 U (1/87 U)
Phenylacetyl	2.3 μ mol/mg (1/1 U)	2.2 μ mol/mg (1/1 U)	
Penamaldate	0.04 μ equiv./mg (1/58 U)	0.03 μ equiv./mg (1/77 U)	1/59.5 U
NMR, aromatic proton to dimethyl proton	5:6	5:6	5:6

* Reported by Smith and Marshall⁶.



Fig. 3. TLC of fractions A, B and C. Plate, Kieselgel 60F₂₅₄; solvent, ethyl acetate-acetic acid-water (15:1:1).

2b). The fraction eluted between K_{av} 0.0 and 0.3 was collected and desalted to give fraction A. For comparison, the fractions eluted between K_{av} 0.3 and 0.55 and between 0.55 and 0.75 were also collected and designated fractions B and C, respectively. In rats sensitized with mouse anti-PcG preparation IgE antibody¹⁸, 1 μ g of each fraction was found to elicit passive cutaneous anaphylactic reactions.

Fraction A developed as a long tailing spot on TLC, whereas B and C exhibited many spots (Fig. 3). From these results, fractions A, B and C were considered to be mixtures of many components. This was confirmed by HPLC using a linear gradient elution technique (Fig. 4). A and B were composed of so many substances that it was difficult to isolate each one by HPLC.

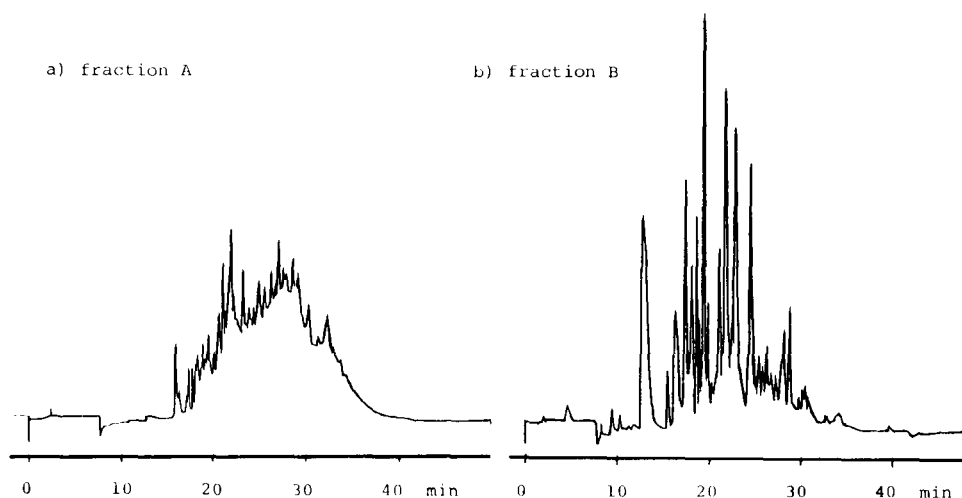


Fig. 4. Reversed-phase HPLC of fractions A and B with linear acetonitrile gradient elution. Column, Toyo Soda LS-410 (5 μ m) (250 \times 4 mm I.D.); eluent, 0.01 *M* phosphate buffer (pH 7.9)-acetonitrile (the acetonitrile concentration was increased from 0 to 50% at the rate of 1%/min); detection, UV absorption at 254 nm.

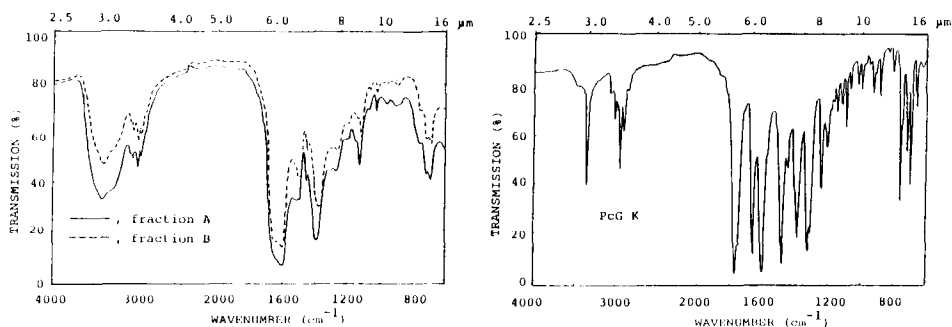


Fig. 5. IR spectra of fractions A and B and PcG potassium (potassium bromide disk).

Cleavage of the β -lactam ring makes it possible for two of the three asymmetric carbons in PcG to racemize¹⁹. Therefore, there are four isomers in monomers and sixteen isomers in dimers. In this manner, the increase in the degree of polymerization results in a marked increase in the number of isomers. This may be one of the reasons for the complexity of the polymer fractions.

The analytical data for A and B are listed in Table I together with those reported by Smith and Marshall⁶. The C:N:S ratios of A and B are similar to that of PcG (16:2:1). The sulphur contents (2.3 $\mu\text{mol}/\text{mg}$) of A and B coincide with the phenylacetyl contents (2.3 $\mu\text{mol}/\text{mg}$ for A and 2.2 for B). Further, the ratios of phenyl proton to dimethyl proton determined by ¹H NMR are 5:6 for both fractions. These data suggest that the ratio of phenylacetyl to thiazolidine and/or penicillamine is 1:1, and that elimination of the fragment composed of the PcG skeleton did not occur appreciably in the polymerization reaction.

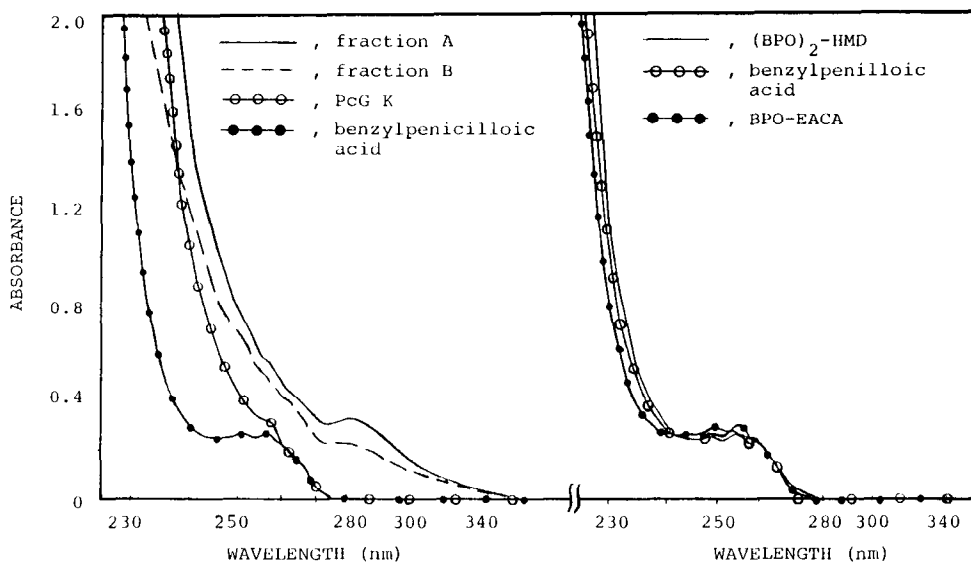


Fig. 6. UV spectra of fractions A and B and PcG-related compounds. Solvent, 0.01 *M* phosphate buffer (pH 7.4); concentration, 2 mg of sample per 5 ml of buffer.

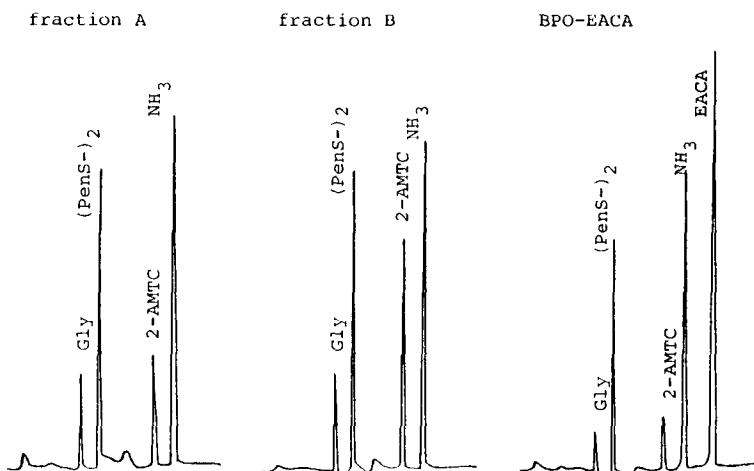


Fig. 7. Amino acid analysis of acid hydrolysates of fractions A and B and BPO-EACA.

The IR spectra of A and B (Fig. 5a) were apparently identical and did not show the absorption (1775 cm^{-1}) of β -lactam seen in the spectrum of PcG (Fig. 5b), suggesting that the chemical structure of A resembled that of B and that both fractions had no β -lactam ring. Their UV spectra (Fig. 6) showed weak maxima at 320 and 280 nm, whereas some of the PcG related compounds did not, indicating the presence of penicillenate (maximum 320 nm) and penamaldate (maximum 280 nm). The penamaldate contents calculated from the molar absorptivity of penamaldate (about $20,000^1$) were $0.04\text{ }\mu\text{equiv./mg}$ for A and $0.03\text{ }\mu\text{equiv./mg}$ for B, suggesting that the ratios of penamaldate to phenylacetyl or thiazolidine (penicillamine) were 1:58 for A and 1:77 for B. Consequently, the penamaldate structure in the polymer could be neglected, as could the penicillenate structure. The thiol contents were $0.025\text{ }\mu\text{equiv./mg}$ for A and $0.06\text{ }\mu\text{equiv./mg}$ for B, indicating that 1/92 or 1/38 of the sulphur molecules were thiol. Therefore, the presence of the thiol group could also be neglected. All these data strongly supported the proposed structure I.

The non-acylated thiazolidine contents¹⁷ of A and B were 0.22 and 0.71 $\mu\text{equiv./mg}$, respectively. The degree of polymerization obtained by dividing the sulphur content ($2.3\text{ }\mu\text{mol/mg}$) by these values was 10 for A and 3.2 for B, and the average molecular weight was calculated to be 3600 for A and 1100 for B. The degree of polymerization of A was found to be larger than that of the polymer reported by

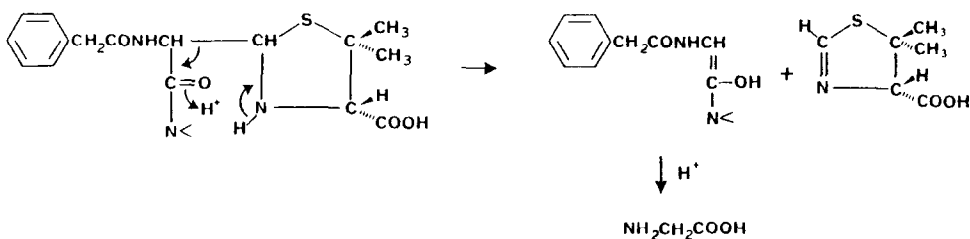


Fig. 8. Formation of glycine from BPO-amines or imines by acid hydrolysis.

Smith and Marshall⁶ (Table I), although both fractions were prepared by almost identical procedures. Dialysis of A after chromatography on Sephadex might lead to the loss of the polymers having low molecular weights⁴.

Acid hydrolysis of A and B yielded glycine, penicillamine disulphide, 2-AMTC, ammonia and a few unidentifiable degradation products (Fig. 7). Hydrolysis of PcG, benzylpenilloic acid, benzylpenicilloic acid and ABPC under the same conditions afforded penicillamine disulphide and 2-AMTC with a minor amount of glycine. BPO-EACA and (BPO)₂-HMD produced penicillamine disulphide and 2-AMTC with a considerable amount of glycine. Consequently, the decomposition reaction shown in Fig. 8 may partly occur in the course of acid hydrolysis of the polymers, BPO-EACA and (BPO)₂-HMD. In the hydrolysis of PcG, benzylpenicilloic acid and ABPC, the precedence of the decarboxylation at C₇ might result in low glycine production. The C₇ in the penicillin skeleton of the polymers therefore seemed partly to form the imido bond as in I.

Fraction C showed five major spots, C-1, C-2, C-3, C-4 and C-5, on TLC (Fig. 9), which were purified by silica gel column chromatography. C-1, C-2 and C-4 were identified as N-formylpenicillamine (II), benzylpenilloic acid (III) and benzylpenillic acid (V), respectively (Fig. 10), by comparing their ¹H NMR and IR spectra and TLC R_F values with those of authentic samples. C-3, which was difficult to purify because it had tended to decompose under the chromatographic conditions, was identified as benzylpenicilloic acid by comparing its R_F values with those of the authentic standard using several solvent systems and colour reagents. C-5 was too small to investigate. In the course of purifying C-4, we obtained a compound that exhibited a tailing spot on TLC and was intensely coloured by DTNB. Its ¹H NMR spectrum gave signals at δ 8.10 (s, 1H), 7.30 (s, 5H), 4.96 (s, 1H), 4.2 (d, 2H), 1.55 (s, 3H) and 1.15 (s, 3H), corresponding to =CH-N, phenyl, CH-CO, CH₂, CH₃ and CH₃, respectively. Its ¹³C NMR spectrum showed the carbon signals of C=N (δ 148.7), =CH- (125.8), =C-COOH (137.0, 163.5), -CH-COOH (66.9, 168.6), C-S (46.7),

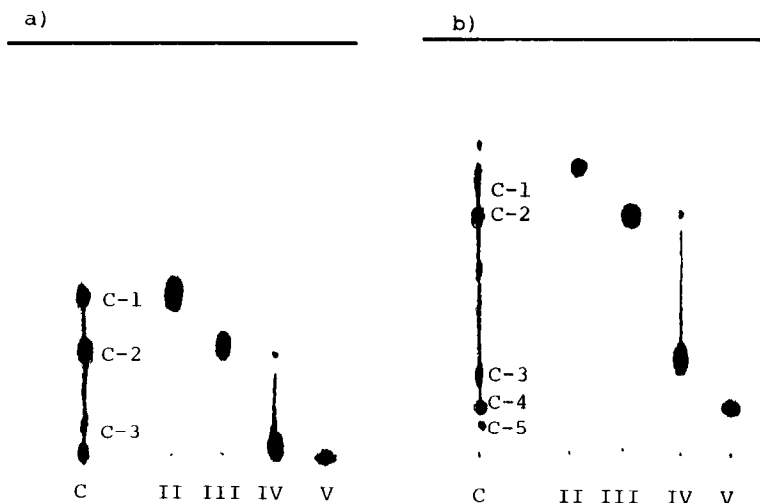


Fig. 9. TLC separation of fraction C and some PcG degradation products. Plate, Kieselgel 60F₂₅₄; solvent, (a) ethyl acetate-acetic acid-water (15:1:1) and (b) ethyl acetate-acetic acid-water (3:1:1).

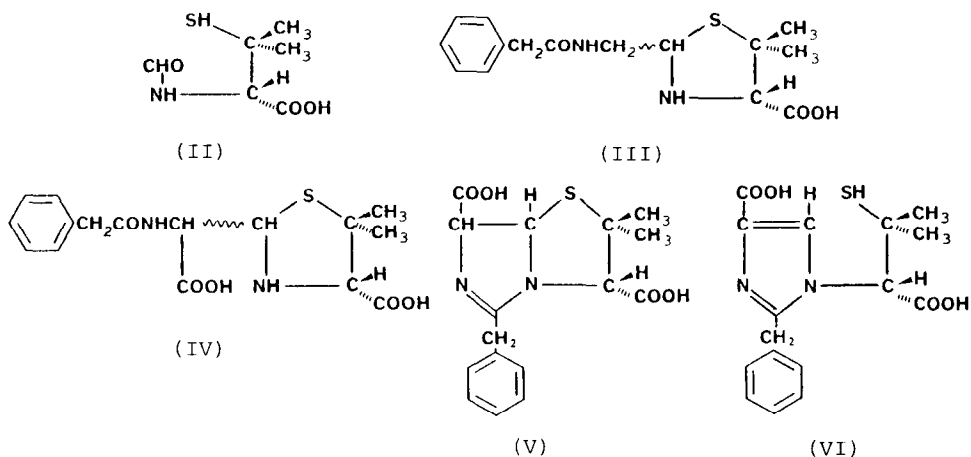


Fig. 10. PcG degradation products found in fraction C.

CH_3 (29.7, 31.6), CH_2 (32.7) and C_6H_5 . Based on these results, together with the elemental analysis data, this compound was assumed to be benzylisopenillic acid (VI). Whether VI was actually present in fraction C or was an artifact produced from V in the purification steps was not clear. In the hydrochloric acid extract of fraction C, penicillamine and its disulphide were not detected by amino acid analysis. From these results, it became clear that the PcG degradation products were mainly eluted from Sephadex G-25 in C and the possibility of the presence of PcG polymers larger than dimers in this fraction was small.

The eluent for the separation of polymers on Sephadex should be buffer or saline because low-molecular-weight substances are sometimes excluded with water in low K_{av} regions³. In this study, we therefore used 0.5% sodium chloride solution as the eluent. Nevertheless, IV and V, of substantially the same molecular weight as PcG, and II and III, of lower molecular weight than PcG, were eluted in lower K_{av} regions than PcG. Takagi *et al.*²⁰ recently reported that some penicillins were strongly adsorbed on Sephadex G-25, whereas penilloic acids were excluded from the gel faster than the parent penicillin. In high-speed gel filtration chromatography²¹, the retention time of fraction C was shorter than that of PcG. The separation of penicillin-related compounds has been reported to be affected by gel-solute interactions, such as electrostatic attraction or repulsion, and adsorption on the gel matrix, in addition to molecular sieving mechanisms²². Hence mechanisms other than molecular sieving caused a disturbance of the correlation between the molecular weight and the elution volume. In studies on the allergenicity of β -lactam antibiotic polymers, compounds having elution volumes smaller than those of the parent antibiotics on gel filtration have been regarded as polymers. Our results showed that polymers should be confirmed by several analytical methods.

This study showed that the polymer fractions isolated by a widely used method contained many components. To clarify the polymer structure further, it is important to investigate the conditions under which polymers of low molecular weight, such as dimers, are effectively produced. Precise studies on the ability of A, B and C to evoke allergic reactions are now in progress.

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