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Structures of Polymers formed in Aqueous Solutions of Cefsulodin Sodium

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Polymers of cefsulodin sodium (CFS) formed in aqueous solution were isolated by Sephadex G-25 gel filtration and purified by dialysis in Visking tubes. Their structures were investigated chemically and by ultraviolet, infrared and nuclear magnetic resonance spectroscopy. From the results, the following polymerization process is suggested: Opening of the β-lactam ring of CFS is followed by decarboxylation, and successive cleavage of the thiazine ring results in the formation of 2-(α-sulfophenylacetamido)acetaldehyde. Aldol condensation of this aldehyde may produce polymers. The polymerization may be terminated by reaction with the isonicotinamide group of CFS. This polymerization process is different from those of penicillins previously reported by several research groups.

Keywords——cephalosporin; cefsulodin sodium; polymerization; structure; physicochemical properties

Among β-lactam antibiotics, penicillins have been a target of intensive investigation on the degradative formation of polymers, which are suspected to cause anaphylaxis, the most serious adverse reaction to these drugs. Several structures have been proposed for penicillin polymers, and the disposition to form polymers has been well correlated with the frequency of allergy outbreak.[1)] In the case of cephalosporins, the stabilities of the drug molecules are rather different from those of penicillins and, moreover, differ from one cephalosporin to another. Consequently the mechanism of polymer formation is so complicated that no probable structure has been proposed as yet.[2)] Work on cephalosporin polymers has been limited, to our knowledge, to measurements of physicochemical properties of the polymer fraction or to studies on the role of substituents at the C-3 position in polymer formation.[3)]

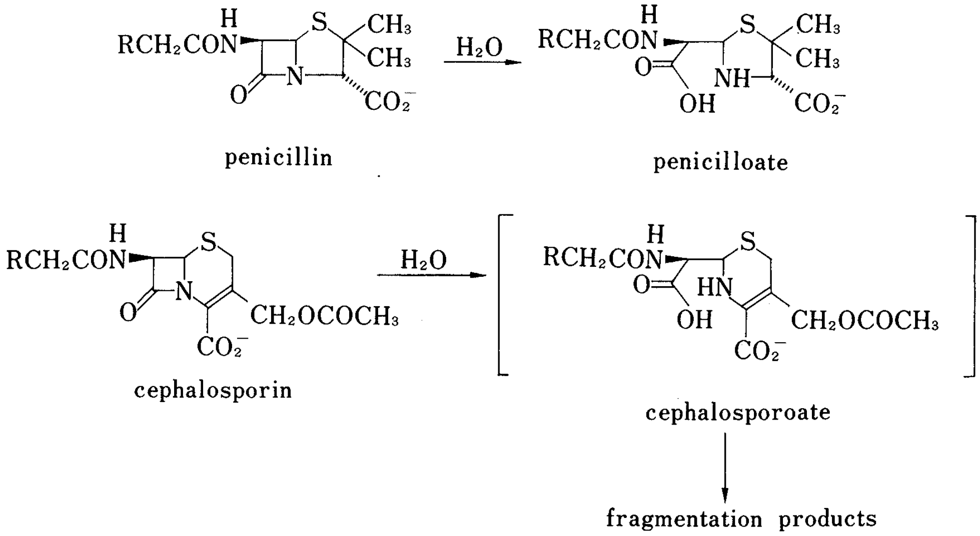


Chart 1

In the hydrolysis of penicillins, the β-lactam ring is primarily opened to yield the penicilloate, which is often isolated as a stable compound. A similar nucleophilic attack on the cephem ring results in diverse reactions, and cephalosporoate produced as an intermediate is too labile to isolate (Chart 1).[4] We recently investigated the degradation and polymer formation of a new semisynthetic cephalosporin, cefsulodin sodium (CFS), which has specific activity against *Pseudomonas*,[5] and obatined some information on the polymer structure and the mechanism of polymer production in aqueous solution.

In aqueous solution, some of the cephalosporins having a tetrazol-5-ylthiomethyl group at the C-3 position of the cephem ring are known to undergo hydrolysis of this group prior to cleavage of the β-lactam ring.[6] In the case of CFS,[7] the product of such a reaction, the 3-hydroxymethyl derivative, was not detected in its decomposition mixture even at an early stage of degradation, and thus opening of the β-lactam ring was assumed to proceed first, just as in the case of penicillins. We isolated a bimolecular reaction product of CFS from the solution and elucidated its structure. The finding of this product, together with physicochemical properties of the polymer, led us to propose a structure for the polymer.

Experimental

Sample——CFS was synthesized in this division. Its physicochemical properties have been reported in other papers.[7]

Chromatographic Columns——Sephadex columns (100 × 1.5 cm i.d.) were prepared by packing with Sephadex G-10 (40—120 μm), G-15 (40—120 μm), G-25 (20—80 μm) or G-50 (50—150 μm). The flow rate was adjusted to about 66 ml/hr when 0.5% NaCl was used as the eluent. The total bed volume of these columns was about 170 ml and the void volumes were determined to be 63, 61, 74 and 57 ml, respectively, using Blue Dextran 2000 as a high polymer standard.

For the isolation of polymer fractions, a wider bore column (100 × 3.0 cm i.d.) was used. Sephadex G-25 (20—80 μm) was swollen with water and packed in the column, the flow rate being 72 ml/hr with water. The total bed volume and void volume were 750 and 310 ml, respectively.

To make a reversed-phase column, about 70 g of LiChroprep RP_{18} (25—40 μm, E. Merck) was suspended in 300 ml of chloroform and packed in a pressure-resistant glass column (50 × 1.7 cm i.d.) under a pressure of 30 kg/cm^2. The column was washed successively with chloroform, methanol and water before use. The flow rate of water was 2 ml/min.

Dialysis——About 10 ml of the sample solution was poured into a Visking tube, 2.5 cm wide and 10 cm long, and dialyzed against a stream of water at room temperature for 16 hr.

Analytical Method——A Waters Associates model 6000A high performance liquid chromatograph was used with a detector, model 440 (UV_{254}). A Toyo Soda LS-410 column (15 × 0.4 cm i.d.) was used with 1% $(NH_4)_2SO_4$ and CH_3CN (47: 3, v/v) as the eluent. The flow rate was 3 ml/min.

Thin-layer chromatography (TLC) was conducted on a plate precoated with Kieselgel 60 F_{254} (E. Merck). As the developing solvent, a mixture of acetone, AcOH, H_2O and NH_4OH (12: 5: 3: 4, v/v) was used. Spots were detected under ultraviolet (UV) light (254 nm) or by exposure to iodine vapor.

Fractionation of Polymers——About 4 ml of 25% CFS aqueous solution (pH 4.1) aged at 37° for 72 hr was applied to the Sephadex G-25 column (100 × 3.0 cm i.d.) and eluted with water. The eluate was monitored with a Uvicord II, model 8300, at 254 nm and the five fractions obtained, C-1, C-2, C-3, C-4 and C-5, were lyophilized separately. The dried residues of C-1, C-2 and C-3, 100 mg each, were redissolved in 10 ml of water and dialyzed. C-4 was applied to a reversed-phase column after concentration and eluted with water. With the aid of an RI monitor, model R-401 (Waters Associates), two peaks were separately collected and freeze-dried.

Measurement of UV, Infrared (IR) and Nuclear Magnetic Resonance (NMR) Spectra——UV spectra were recorded with a Perkin-Elmer 554 spectrophotometer and IR spectra with a Perkin-Elmer 218B spectrophotometer. ^1H- and ^{13}C-NMR spectra were measured with a Varian XL-100-12 spectrometer at 100 MHz with tetramethylsilane as an external standard and at 25 MHz with dioxane as an internal standard, respectively.

Results and Discussion

Fractionation of Polymers

The chromatogram obtained with the Sephadex G-25 column is illustrated in Fig. 1, showing the fractions C-1 to C-5. Each fraction was analytically chromatographed on a Sephadex

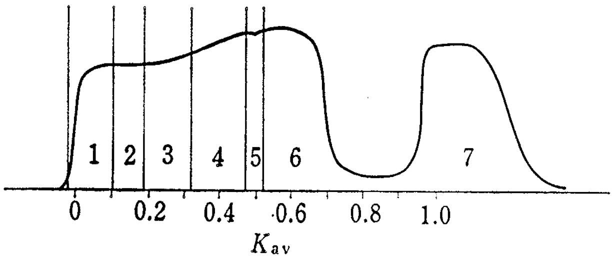


Fig. 1. Preparative Gel Filtration Chromatogram of 25% CFS Aqueous Solution (37°, 72 hr)

1, C-1; 2, C-2; 3, C-3; 4, C-4; 5, C-5; 6, CFS; 7, isonicotinamide.

G-25 column (100×1.5 cm i.d.) and gave the results shown in Fig. 2. Fraction C-5 was found to be composed mostly of unreacted CFS. The other fractions were reapplied to Sephadex G-10 and G-15 columns and their molecular weights were deduced from the retention times to be 4000—4500 for C-1, about 3000 for C-2, about 1500 for C-3 and about 700 for C-4. The last fraction was assumed to be a bimolecular reaction product on the basis of its molecular weight compared with that of CFS (mol. wt. 554.52).

Purification and Structure Elucidation of C-4

TLC examination of C-4 showed that it contained CFS (Rf=0.41) and various related compounds in addition to the main spot (Rf=0.49) (Fig. 3). By high performance liquid chromatography (HPLC), the main spot on TLC was revealed to consist of overlapping spots of two components, C-4A and C-4B (Fig. 4), in nearly equimolecular amounts. With a column

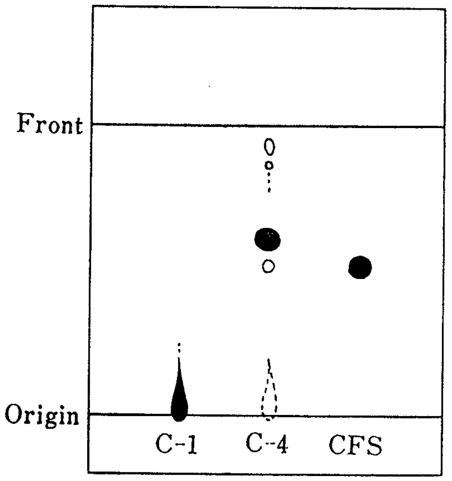


Fig. 3. Thin-Layer Chromatograms of C-1, Crude C-4 and CFS

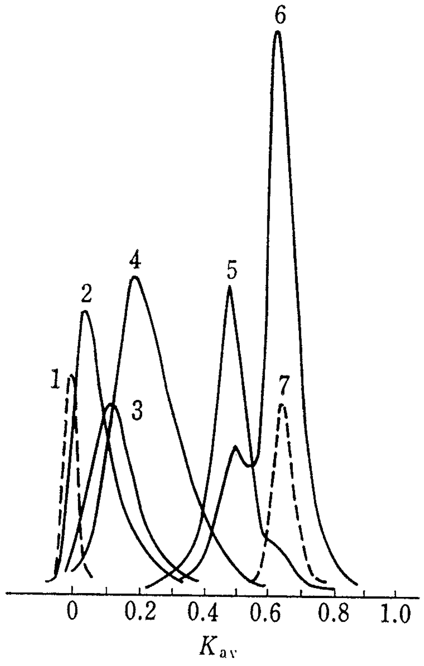


Fig. 2. Analytical Gel Filtration Chromatograms of CFS Polymer Fractions

1, Blue Dextran 2000; 2, C-1; 3, C-2; 4, C-3; 5, C-4; 6, C-5; 7, CFS.

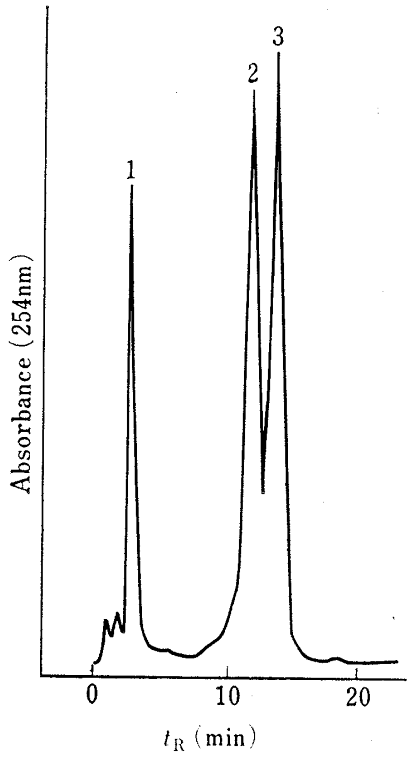


Fig. 4. Reversed-phase High Performance Liquid Chromatogram of Crude C-4

1, CFS; 2, C-4A; 3, C-4B.

of LiChroprep RP_{18}, these two components were isolated in thin-layer chromatographically pure states (Fig. 5). From the results of HPLC, it was evident that the C-4A and C-4B fractions were mutually contaminated with small amounts of each other. The purities of the C-4A and C-4B fractions were estimated to be 99.0 and 99.9% on the basis of peak-area ratios, respectively. The infrared spectra of both components were apparently identical (Fig. 6). In the UV spectra, they showed a maximum at the same wavelength, $\lambda=262$ nm, and $E^{1\%}_{1\text{cm}}$ was 154 for C-4A and 150 for C-4B (Fig. 7). The slight difference in absorption intensity may not be significant in view of the fluctuation of water contents in the samples, which were obtained by lyophilization. The difference in optical rotating power, $[\alpha]^{20}_{D}$ ($c=1$, H_2O) $+18.3$ for C-4A and $+1.5$ for C-4B, indicated that they may be diastereomers.

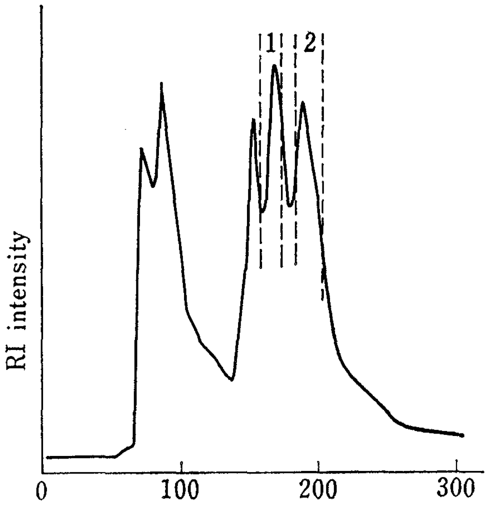


Fig. 5. Separation of Crude C-4 on a LiChroprep RP_{18} Column
1, C-4A; 2, C-4B.

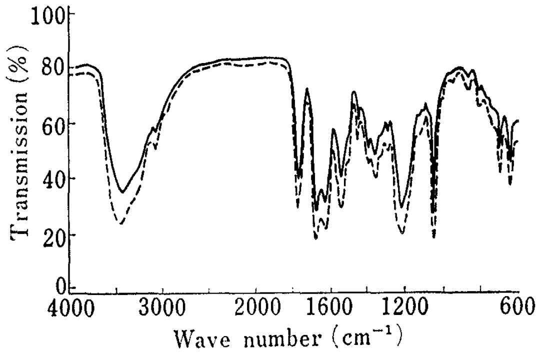


Fig. 6. IR Spectra of C-4A and C-4B (KBr Disk)
----, C-4A; ——, C-4B.

In the ^{1}H-NMR spectrum of C-4B, the signals of C–$\underline{H}$ protons were in accord with those of CFS except that the aromatic proton at the β-position of isonicotinamide was shifted to δ 7.96 in C-4B from δ 8.36 in CFS. Additional signals in C-4B were due to –C$\underline{H}$(SO_3Na)– at δ 4.98 (s), five aromatic protons between δ 7.1 and 7.7 and ABX type protons, –$\dot{C}\underline{H}$–C$\underline{H}_2$– at δ 5.67 (dd, 1H) and δ 3.67 (m, 2H), as listed in Table I. No signals for amino protons corresponding to –N$\underline{H}_2$ in the isonicotinamide moiety of CFS were detected even in d_6-DMSO solution. The ^{13}C-NMR spectrum was consistent with the above results. Twenty-two carbons belonging to 18 differentiated groups in CFS were all located in the spectrum of C-4B. In addition, one carbon assignable to –CH(SO_3Na)–$\underline{C}$ONH– was observed at δ 169.5, six carbons assignable to a benzene ring at δ 129.4 (2C), δ 129.6 (1C), δ 130.2 (2C) and δ 132.4 (1C) and two carbons assignable to $-\overset{|}{N}-\underline{C}H_2-\underline{C}H(OH)-\overset{|}{N}-$ at δ 43.6 and δ 74.1 (Table II). Based on these results together with the elemental analysis data listed below, the structure of C-4B was assumed to

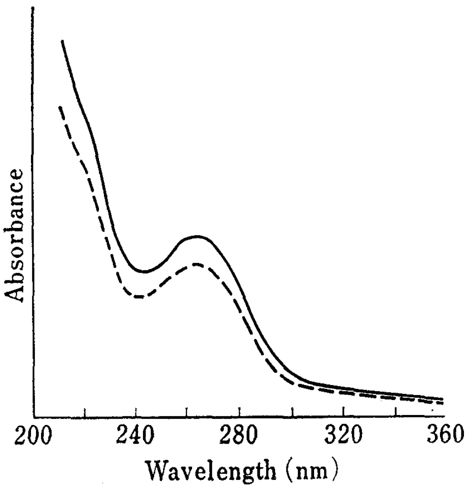


Fig. 7. UV Spectra of C-4A and C-4B in Water
----, C-4A; ——, C-4B.

TABLE I. ^{1}H-NMR Assignments of C-4B in D_2O

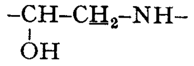
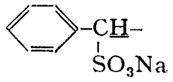
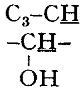
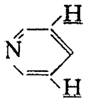
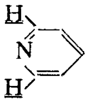
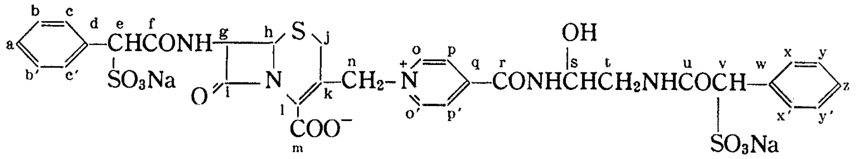
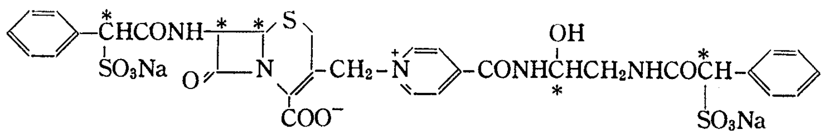
Chemical shift (δ, ppm)	Intensity ratio	Assignment (proton as)
3.32	2	$C_2\text{-}\underline{H}_2$
3.67	2	 $\underset{\text{OH}}{\text{-CH}}\text{-C}\underline{\text{H}}_2\text{-NH-}$
4.98	1	 $\text{-CH}_2\text{NHCOC}\underset{\text{SO}_3\text{Na}}{\underline{\text{H}}}\text{-}$
5.09	1	
5.23	1	$C_6\text{-}\underline{H}$
5.47	2	$C_3\text{-C}\underline{H}_2\text{-}$
5.67	1	 $\text{-C}\underset{\text{OH}}{\underline{\text{H}}}\text{-}$
5.76	1	$C_7\text{-}\underline{H}$
7.10—7.70	10	Aromatic protons
7.96	2	
9.01	2	

TABLE II. ^{13}C-NMR Chemical Shifts for **1** in D_2O



Carbon No.	Chemical shift (ppm)	Carbon No.	Chemical shift (ppm)
u	169.5	a, z	129.6
f	169.5	b, b′, y, y′	129.4
i, m, r	167.4 165.2 164.5	p, p′	126.9
		s	74.1
		v	71.3
q	148.9	e	70.6
o, o′	146.1	n	62.7
k, l	135.8 114.0	h	60.5
		g	58.3
d	132.8	t	43.6
w	132.4	j	25.8
c, c′, x, x′	130.2		



1

$C_{32}H_{29}N_5Na_2O_{13}S_3$, mol wt. 833.77
*: asymmetric carbon

Chart 2

be **1** (Chart 2). C-4A Anal. Calcd for $C_{32}H_{29}N_5Na_2O_{13}S_3 \cdot 5.5\ H_2O$: C, 41.20; H, 4.32; N, 7.51; S, 10.31. Found: C, 40.62; H, 4.52; N, 7.75; S. 10.70. C-4B Anal. Calcd for $C_{32}H_{29}N_5Na_2O_{13}S_3 \cdot 5.5\ H_2O$: C, 41.20; H, 4.32; N, 7.51; S, 10.31. Found: C, 41.14; H, 4.58; N, 7.45; S, 10.76. C-4A was assumed to be a diastereomer of C-4B from the agreement of the spectral data, including that from ^{1}H-NMR, and the difference in optical rotation. Since there are five asymmetric carbons in the structure of **1**, it is difficult to say which carbon is responsible for the optical activity, but considering the process leading to **1** and the relative amounts of C-4A and C-4B (see Fig. 4), a configurational difference at –NH–C*H(OH)– seems most probable. As is already known, cephalosporoate formed by cleavage of the β-lactam moiety in cephalosporins is very unstable, and the thiazine ring is successively ruptured to give an aldehyde. A similar reaction may have occurred in the case of CFS, and the resulting aldehyde **2** may have condensed with the $-CONH_2$ group on another CFS molecule to produce **1** (Chart 3).

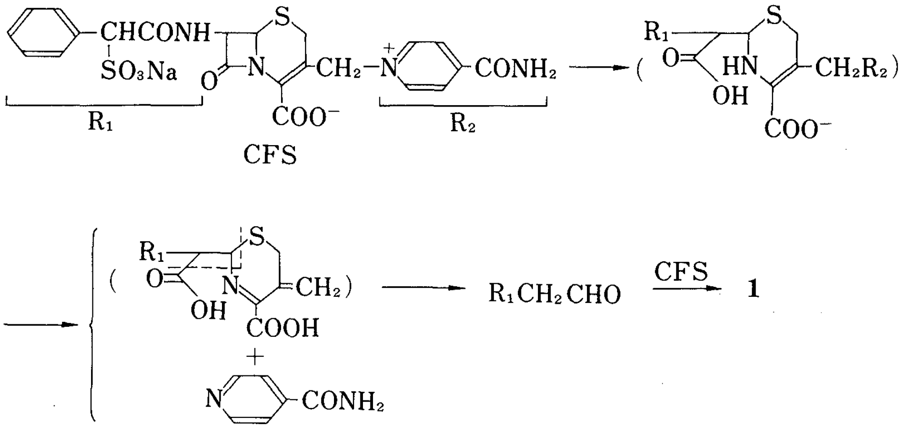


Chart 3

Structure of C-1

The highest polymer fraction, C-1, was dialytically purified in order to eliminate small-molecular contaminants, and subjected to spectral analysis. In the infrared spectrum, the carbonyl stretching vibration band of the β-lactam ring was observed at 1770 cm^{-1}, though the intensity was only about one-fifth of that of CFS. This band disappeared when C-1 was dissolved in 0.1 N sodium hydroxide solution followed by lyophilization, which supported the view that cleavage of the β-lactam ring had occurred. The UV maximum of C-1 also coincided with that of CFS, but the intensity was about one-eighth of that of CFS when expressed in terms of $E^{1\%}_{1\text{cm}}$ (Table III).

Table III. Absorption Intensities of C-1, C-4A, C-4B and CFS at 262 nm

Compound	$E^{1\%}_{1\text{cm}}$ (262 nm)	Ratio (%)
C-1	34	12.5
C-4A	154	56.4
C-4B	150	54.9
CFS	273	100.0

By HPLC, a small amount of isonicotinamide was detected in the solution of C-1 in 0.1 N sodium hydroxide after it had been left to stand for 10 min at room temperature. This may indicate the existence of the isonicotinamido group in an inner part of the structure of C-1. Antibacterial activity of C-1 against *Pseudomonas aeruginosa NCTC* 10490 was weak but de-

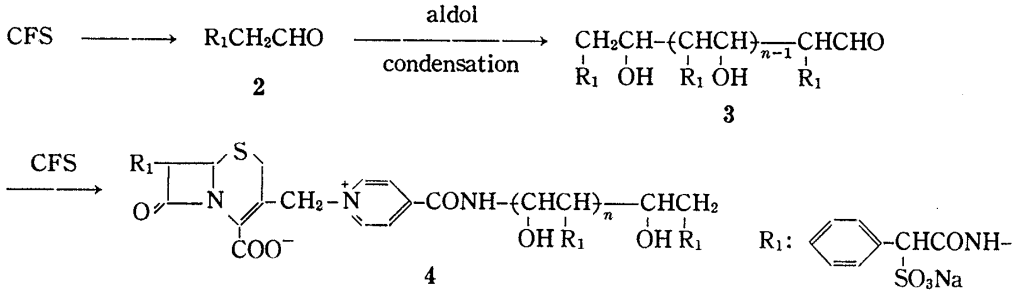


Chart 4

finite, suggesting the presence of CFS structure in C-1. From these results, C-1 was assumed to be formed by the mechanism shown in Chart 4. The aldehyde **2** formed by hydrolysis may polymerize to **3** and finally combine with CFS to afford **4**. If the average molecular weight of C-1 is supposed to be 4500 daltons, about 14 molecules of **2** participated in aldol condensation and the polymer **4** ($n=13$) was produced by further condensation with one molecule of CFS. The atomic ratio, C: N: S, based on this structure was calculated to be 9.0: 1.0: 0.9, which was approximately equal to the observed value of 8.7: 1.0: 1.2 found for C-1 by elemental analysis. This evidence may indicate that at least the bulk of the polymer formed from CFS in aqueous solution can be represented by the structure **4**. This structure is considerably different from the structures proposed hitherto for penicillin polymers, and the present work shows that the polymerization processes of penicillins and cephalosporins may differ markedly.

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References and Notes

1) H. Smith and A.C. Marshall, *Nature* (London), **232**, 45 (1971); M.G. DeAngell, G. Mercandalli, F. Minoja, and S. Tedesschi, *Farmaco.*, **35**, 100 (1980).
2) G.T. Stewart, *Antimicrob. Ag. Chemother.*, **1967**, 543.
3) M.K. Staufield, B.L. Warren, and F.H. Wilson, Abstracts of Papers, 174th National Meeting of American Chemical Society, Chicago, Illinois, August, 1979, Coll 130; Y. Takeuchi, N. Hayashi, and Y. Kimura, *Jpn. J. Allergol.*, **28**, 200 (1979).
4) G.V. Kaiser and S. Kukolja, "Cephalosporins and Penicillins Chemistry and Biology," ed. by E.H. Flynn, Academic Press, New York and London, 1972, pp. 125—126.
5) H. Nomura, T. Fugono, T. Hitaka, I. Minami, T. Azuma, S. Morimoto, and T. Masuda, *J. Med. Chem.*, **17**, 1312 (1974); H. Nomura, I. Minami, T. Hitaka, and Y. Fugono, *J. Antibiōt.*, **29**, 428 (1976).
6) K. Itakura, T. Onoue, M. Godo, Y. Oda, K. Naito, and M. Kuwayama, *Takeda Kenkyusho Nempo*, **37**, 286 (1978).
7) I. Aoki, A. Konishi, and M. Kuwayama, *Iyakuhin Kenkyu*, **9**, 1022 (1978).