Chemical Reactions in Cephalosporin Allergy: High-Pressure Liquid Chromatographic Analysis of Cephalosporin Aminolysis Kinetics

AKIRA TSUJI, ETSUKO MIYAMOTO, and TSUKINAKA YAMANA ˣ

Received May 2, 1978, from the *Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan.* Accepted for publication October 30, 1978.

Abstract □ Cephalosporin reaction with protein amino groups is fundamental to cephalosporin allergy. Cephalothin and cefazolin reaction kinetics with ε-aminocaproic acid, β-alanine, and glycine in aqueous solution were investigated. All reactions were conducted at 35° and 0.5 ionic strength and were followed by ion-exchange high-pressure liquid chromatography. The aminolysis rate constants can be expressed as terms representing uncatalyzed or water-catalyzed amine reaction, self-assisted nucleophilic reaction, and hydroxide-ion-catalyzed nucleophilic amine attack on the β-lactam moiety. Cephalothin and cefazolin react with amines as readily as penicillin G. The UV spectra of several cephalothin–glycine reaction products were recorded, and their possible structures are discussed.

Keyphrases □ Cephalosporins—aminolysis kinetics, allergy, high-pressure liquid chromatography □ Aminolysis—cephalosporins, allergy, kinetic analysis, high-pressure liquid chromatography □ Kinetics—cephalosporin aminolysis, high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, cephalosporins, aminolysis kinetics, aminolysis products □ Antibacterials—cephalothin, deacetylcephalothin, and cefazolin, aminolysis kinetics, cephalosporin allergy, high-pressure liquid chromatography

Since cephalosporin reaction with free amino groups on tissue proteins to yield cephalosporoyl conjugates *in vivo* may be largely responsible for cephalosporin immunogenicity (1), a knowledge of cephalosporin aminolysis and reaction products is important. Such protein conjugates prepared *in vitro* have been strongly immunogenic in animals (2–6).

Although kinetic studies on penicillin aminolysis have been reported (7–17), little work has been performed using cephalosporins, probably due to analysis difficulties, reaction complexity, and the dearth of information on these compounds. Cephalosporin aminolysis kinetic analysis by differential UV spectrophotometry was reported recently (18, 19). Spectrophotometric analysis has been useful for following cephalosporin hydrolysis (18–23).

This paper reports the high-pressure liquid chromatographic (HPLC) analysis of cephalosporin aminolysis kinetics. This method seems to be a powerful tool for quantifying reactions, separating the hydrolysis and aminolysis products, and investigating degradation products. HPLC analysis of the kinetics and mechanism of aqueous cephalosporin hydrolysis as a function of pH was reported previously (23, 24).

EXPERIMENTAL

Materials—Cephalothin sodium[1] (944 μg/mg), deacetylcephalothin sodium[1], and cefazolin sodium[2] (966 μg/mg) were used as received.

All other chemicals were the highest grade and were used without further purification.

Kinetic Procedure—Three amino acid types, glycine, β-alanine, and ε-aminocaproic acid, were used both as buffers to maintain a constant pH and as nucleophiles. The amine buffers were prepared just before use with calculated amounts of amino acid and standard sodium hydroxide solution. The ionic strength was adjusted to 0.5 with potassium chloride. The kinetic technique was almost the same as one used previously for penicillin aminolysis (12–14).

To avoid the possible change in the kinetic solution pH[3] during the reaction, a constant pH was maintained with a pH-stat[4]. All reactions were conducted at 35.0 ± 0.1° with the total amine concentration in large excess over the reacting substrate concentration to maintain pseudo-first-order kinetics. In most cases, the initial cephalosporin concentration was 5×10^{-3} *M* and the total amine concentration was 0.04–0.60 *M*. Reaction solution aliquots (1 ml) were withdrawn at intervals, neutralized to quench the reaction by addition of 1 ml of 0.2 *M* phosphate buffer (pH 6.0) in a cooled water bath to give the final pH of 7–8, and analyzed by HPLC as described later.

Table I—Apparent First-Order Rate Constants, k_{obs}, for Cephalothin Aminolysis in Various Amine Buffers at 0.5 Ionic Strength and 35°

Buffer			pH[a]	k_{obs}, hr^{-1}
$[NH_3^+CH_2COO^-]$	[NaOH]	[KCl]		
0.060	0.033	0.467	9.50	0.274
0.120	0.066	0.434	9.50	0.499
0.060	0.043	0.457	9.80	0.533
0.120	0.088	0.412	9.84	1.11
0.180	0.131	0.369	9.84	1.86
0.060	0.044	0.456	9.85	0.638
0.120	0.096	0.404	10.00	1.28
0.180	0.143	0.357	10.00	2.24
0.300	0.239	0.261	10.00	4.94
$[NH_3^+(CH_2)_2COO^-]$	[NaOH]	[KCl]		
0.060	0.020	0.480	9.70	0.460
0.380	0.131	0.369	9.70	2.85
0.600	0.207	0.293	9.70	6.11
0.038	0.019	0.481	10.00	0.830
0.075	0.038	0.462	10.00	1.10
0.150	0.077	0.423	10.00	1.95
0.300	0.150	0.347	10.00	4.50
0.050	0.033	0.467	10.30	1.90
0.080	0.054	0.446	10.30	2.37
0.100	0.068	0.432	10.30	2.89
0.200	0.135	0.365	10.30	5.61
0.300	0.203	0.297	10.30	9.25
0.050	0.038	0.462	10.50	3.00
0.060	0.046	0.454	10.50	3.26
0.150	0.115	0.385	10.50	6.25
0.200	0.153	0.347	10.50	8.20
$[NH_3^+(CH_2)_5COO^-]$	[NaOH]	[KCl]		
0.050	0.011	0.489	10.00	1.09
0.100	0.022	0.478	10.00	1.85
0.150	0.033	0.467	10.00	2.80
0.200	0.044	0.456	10.00	3.90
0.040	0.014	0.486	10.30	2.17
0.100	0.035	0.465	10.30	4.15
0.120	0.042	0.458	10.30	4.91
0.150	0.053	0.447	10.30	6.74
0.020	0.013	0.487	10.80	6.12
0.047	0.030	0.470	10.80	9.76
0.094	0.060	0.440	10.80	17.7

[a] The pH was maintained by a pH-stat.

[1] Shionogi & Co., Osaka, Japan.
[2] Fujisawa Pharmaceutical Co., Osaka Japan.
[3] PHM26 pH-meter, Radiometer, Copenhagen, Denmark.
[4] A pH-stat titrimeter assembly consisting of a TTT11 titrator, a PHM26 pH-meter, and an ABU12b autoburet, Radiometer, Copenhagen, Denmark.

0022-3549/79/0500-0616$01.00/0

Table II—Apparent First-Order Rate Constants, k_{obs}, for Deacetylcephalothin Aminolysis in Glycine Buffer at 0.5 Ionic Strength and 35°

Buffer			pH[a]	k_{obs}, hr^{-1}
$[NH_3^+CH_2COO^-]$	[NaOH]	[KCl]		
0.120	0.066	0.434	9.50	0.185
0.180	0.099	0.401	9.50	0.359
0.060	0.048	0.452	10.00	0.258

[a] The pH was maintained by a pH-stat.

Table III—Apparent First-Order Rate Constants, k_{obs}, for Cefazolin Aminolysis in Various Amine Buffers at 0.5 Ionic Strength and 35°

Buffer			pH[a]	k_{obs}, hr^{-1}
$[NH_3^+CH_2COO^-]$	[NaOH]	[KCl]		
0.080	0.049	0.451	9.60	0.340
0.200	0.122	0.378	9.60	1.40
0.300	0.182	0.318	9.60	2.82
0.120	0.094	0.406	10.00	1.06
0.190	0.153	0.347	10.00	2.28
0.250	0.198	0.302	10.00	3.51
0.300	0.236	0.264	10.00	5.19
$[NH_3^+(CH_2)_2COO^-]$	[NaOH]	[KCl]		
0.060	0.020	0.480	9.70	0.277
0.150	0.052	0.448	9.70	0.790
0.300	0.103	0.397	9.70	1.96
0.380	0.131	0.369	9.70	2.96
0.585	0.201	0.299	9.70	6.29
0.080	0.054	0.446	10.30	1.52
0.150	0.102	0.398	10.30	2.90
0.200	0.135	0.365	10.30	4.40
0.210	0.142	0.358	10.30	4.55
0.300	0.203	0.297	10.30	7.96
0.050	0.040	0.460	10.60	1.96
0.100	0.081	0.419	10.60	3.42
0.168	0.136	0.364	10.60	6.11
$[NH_3^+(CH_2)_5COO^-]$	[NaOH]	[KCl]		
0.050	0.011	0.489	10.00	0.741
0.100	0.022	0.478	10.00	1.46
0.150	0.033	0.467	10.00	2.38
0.217	0.048	0.452	10.00	3.99
0.040	0.014	0.486	10.30	1.53
0.070	0.025	0.475	10.30	2.60
0.120	0.043	0.457	10.30	4.77
0.146	0.052	0.448	10.30	6.00
0.160	0.058	0.442	10.30	7.07
0.200	0.072	0.428	10.30	9.66

[a] The pH was maintained by a pH-stat.

The pseudo-first-order rate constants, k_{obs}, were usually calculated from the slope of the semilogarithmic plots of the residual concentration starting materials *versus* time by the least-squares treatment. Tables I–III summarize the amine buffer compositions and the k_{obs} values.

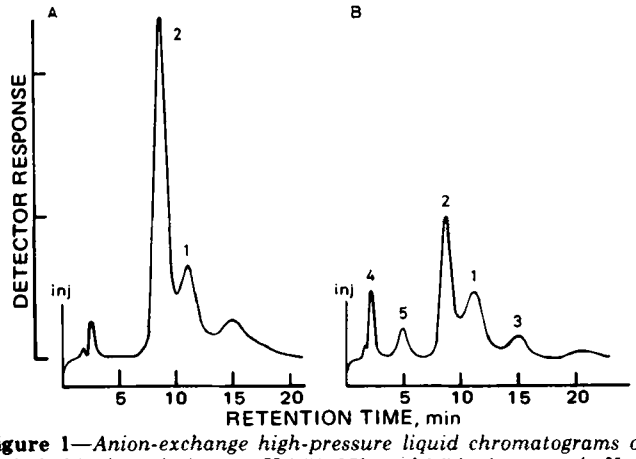


Figure 1—*Anion-exchange high-pressure liquid chromatograms of cephalothin degradation at pH 9.50, 35°, and 0.5 ionic strength. Key: A, sample after 10 hr of hydrolysis; and B, sample after 5 hr of reaction with 0.12 M glycine.*

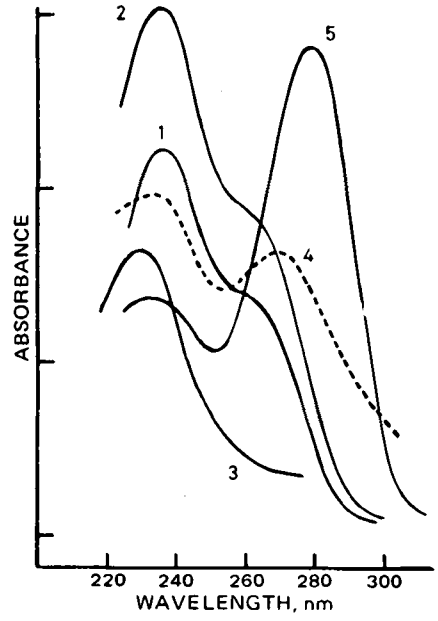


Figure 2—*UV spectra of peaks 1–5 in Fig. 1B.*

HPLC Study—The liquid chromatograph[5] was equipped with a double-beam, variable-wavelength UV detector[6] set at 254 nm. The stationary phase was strong anion-exchange resin prepacked into a stainless steel column, 2.1 mm i.d.[7]. The mobile phases were aqueous 0.02 *M* NaH_2PO_4 adjusted to pH 8.5 with sodium hydroxide for cephalothin and aqueous 0.2 *M* CH_3COONa adjusted to pH 5.0 for cefazolin. The operating parameters for the instrument were: pressure, 40–100 kg/cm^2 (changed to obtain suitable retention time for analysis); temperature, ambient; and UV attenuation, 0.16–0.32 aufs.

A 5-μl sample was injected using a 10-μl syringe and a stopped-flow mode. The cephalothin and deacetylcephalothin or cefazolin content was determined by comparing the peak heights with those of similarly chromatographed standards. Calibration curves for the substances were prepared daily.

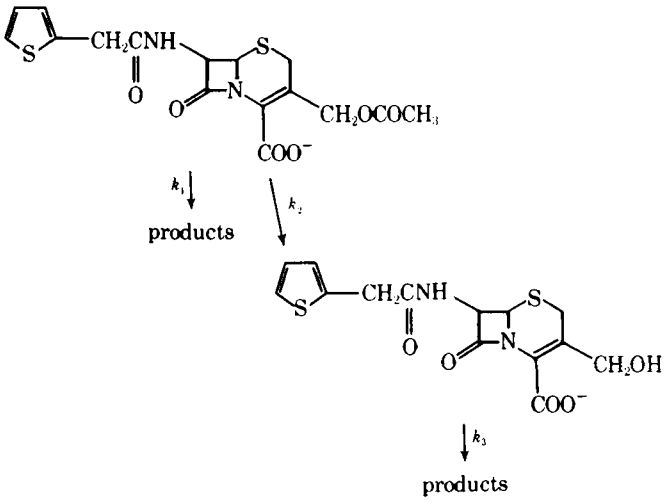
TLC Study—TLC was employed to identify the degradation products and to verify HPLC results. Aliquots (10 μl) of the 0.01 *M* cephalothin–glycine reaction solution were applied to glass plates (5 × 20 cm) precoated with 250-μm silica gel[8]. Chromatograms were run for 15 cm in acetic acid–ethanol–chloroform (1:7:12). Spots corresponding to cephalosporins were visualized with iodine vapor. Authentic cephalothin and deacetylcephalothin samples showed R_f values of 0.39 and 0.18, respectively. The standard *N*-acetylglycine solution showed a spot at R_f 0.36, which was visualized with bromcresol green (18).

RESULTS AND DISCUSSION

Monitoring Hydrolysis and Aminolysis of Cephalothin by HPLC—Figure 1A represents the typical chromatogram of the 10-hr aged hydrolysis solution of cephalothin at 35°, μ = 0.5, and pH 9.5 maintained with a pH-stat. Several peaks appeared; two could be assigned, as demonstrated previously (23), to cephalothin itself (peak 1) and deacetylcephalothin (peak 2) resulting from 3-acetoxy moiety hydrolysis. Others were regarded as β-lactam cleavage products.

The cephalothin hydrolytic pathway in alkaline solution has been established kinetically (18, 23) as represented in Scheme I, where k_1 and k_3 represent the rate constants for cleavage of cephalothin and deacetylcephalothin β-lactam rings, and k_2 is the rate constant for cephalothin deacetylation. Simultaneous and quantitative cephalothin and deacetylcephalothin determination at pH 9.5, 35°, and μ = 0.5 was made by HPLC. Kinetic parameters (Scheme I) were computed by the nonlinear least-squares[9] method to be (in hr^{-1}): k_1 = 0.070, k_2 = 0.137, and k_3 = 0.069. Detailed kinetics (18, 23) and the mechanism (23) of cepha-

[5] Shimadzu-DuPont model 830, Kyoto, Japan.
[6] UV 202 recording spectrophotometer, Shimadzu, Kyoto, Japan.
[7] Zipax SAX, 0.5 or 1 m, DuPont Instruments, Wilmington, Del.
[8] E. Merck, Darmstadt, West Germany.
[9] FORTRAN IV computer program written by the authors. A digital computer, FACOM 230-35, was used at the Data Processing Center, Kanazawa University.



Scheme I

lothin hydrolysis were reported previously. The hydrolysis rate constants for k_1, k_2, and k_3 can be defined, in nonbuffer solutions, as $(k_1)_{pH}$, $(k_2)_{pH}$, and $(k_3)_{pH}$ and obey Eqs. 1*a*–1*c* above pH 9:

$$(k_1)_{pH} = (k_1)_{OH}a_{OH^-} \quad \text{(Eq. 1}a\text{)}$$

$$(k_2)_{pH} = (k_2)_{OH}a_{OH^-} \quad \text{(Eq. 1}b\text{)}$$

$$(k_3)_{pH} = (k_3)_{OH}a_{OH^-} \quad \text{(Eq. 1}c\text{)}$$

where $(k_i)_{OH}$ is the specific hydroxide ion-catalyzed rate constant for the respective hydrolytic reactions and a_{OH^-} is the hydroxide-ion activity and can be calculated at 35° from (25):

$$a_{H^+}a_{OH^-} = 10^{-13.68} \quad \text{(Eq. 2)}$$

The $(k_i)_{OH}$ average values (35°, μ = 0.5) for $(k_i)_{pH}$ from the data determined by HPLC and previously (23), according to Eqs. 1*a*–1*c*, were 1.06 × 10^3, 1.82 × 10^3, and 1.01 × 10^3 M^{-1} hr^{-1}, respectively.

A typical 5-hr chromatogram of cephalothin with 0.12 *M* glycine (pH 9.5, 35°, μ = 0.5) is shown in Fig. 1B. There were some differences in the product peaks between hydrolysis (Fig. 1A) and aminolysis at the same pH. UV spectra of these peaks are shown in Fig. 2. Peaks 1 and 2 had two characteristic absorption maxima at 235 and 260 nm, consistent with those of cephalothin and deacetylcephalothin (26). Peaks 3–5 were assigned to the glycinolysis products of cephalothin and/or deacetylcephalothin. These findings indicate that the cephalothin aminolysis is also represented as Scheme I.

The time course (mole percent) for cephalothin and deacetylcephalothin during cephalothin glycinolysis at pH 9.5 (Fig. 3) showed good agreement with values calculated from the nonlinear least-squares analysis[9]. The rate constants computed were (in hr^{-1}): k_1 = 0.345 ± 0.008, k_2 = 0.161 ± 0.007, and k_3 = 0.184 ± 0.018 (*SD*). No detectable *N*-

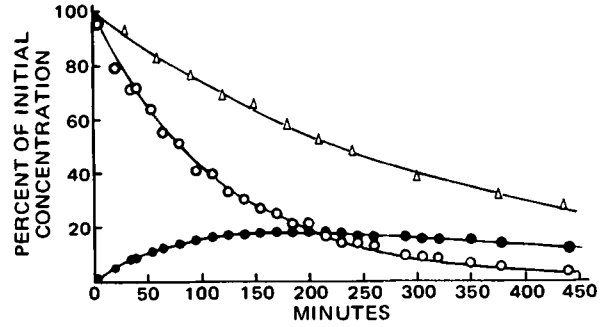


Figure 3—*Time course for cephalothin* (○) *and deacetylcephalothin* (●) *during cephalothin aminolysis and that for deacetylcephalothin aminolysis* (△), *both with 0.12* M *glycine at pH 9.50, 35°, and 0.5 ionic strength. Points for cephalothin aminolysis were from several experiments by HPLC under the same conditions. The curves were calculated according to Scheme I by the use of the following rate constants (in hours*$^{-1}$*):* k_1 = *0.345,* k_2 = *0.164, and* k_3 = *0.184.*

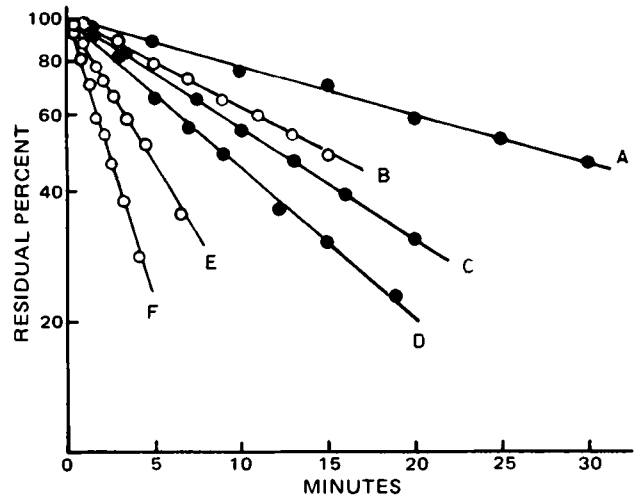


Figure 4—*Apparent first-order plots determined by HPLC for the aminolysis of cephalothin* (○) *and cefazolin* (●) *at 35° and 0.5 ionic strength. Key: A, 0.08* M *β-alanine at pH 10.30; B, 0.38* M *β-alanine at pH 9.70; C, 0.25* M *glycine at pH 10.00; D, 0.12* M *ε-aminocaproic acid at pH 10.30; E, 0.30* M *β-alanine at pH 10.30; and F, 0.094* M *ε-aminocaproic acid at pH 10.80.*

acetylglycine formation was found on TLC, as previously observed (18). These kinetic and TLC results indicate no significant ester moiety aminolysis at the cephalothin 3-position. The deacetylcephalothin reaction in 0.12 *M* glycine buffer (pH 9.5, 35°, μ = 0.5) is shown in Fig. 3. The first-order degradation rate constant was k_3 = 0.183 $hr,^{-1}$ which is in fair agreement with that computed from the cephalothin glycinolysis under the same conditions.

For other aminolysis reactions followed by HPLC, cephalothin disappearance followed first-order kinetics (Fig. 4). The pseudo-first-order rate constant, k_{obs}, can be commonly expressed for the reactions in all amine solutions as:

$$k_{obs} = k_{pH} + k_{amine} \quad \text{(Eq. 3)}$$

where k_{pH} is the cephalothin first-order hydrolysis rate constant and is equal to $(k_1)_{pH}$ + $(k_2)_{pH}$ and k_{amine} is the first-order rate constant depending on the concentration of amines.

Monitoring Cefazolin Aminolysis—Typical chromatograms of cefazolin aminolysis with ε-aminocaproic acid are shown in Fig. 5 as a function of time. Cefazolin aminolysis occurs only at the β-lactam moiety. The semilogarithmic plots of the remaining cefazolin percentage were reasonably linear (Fig. 4), indicating that the cefazolin degradation followed first-order kinetics. The pseudo-first-order rate constant, k_{obs}, obtained from the slopes is the sum of the competing rate constants, k_{pH} for hydrolysis and k_{amine} for aminolysis, and is given by Eq. 3. Cefazolin hydrolysis k_{pH} obeys Eq. 4 above pH 9, as verified previously (18, 23):

$$k_{pH} = k_{OH}a_{OH^-} \quad \text{(Eq. 4)}$$

The value of k_{OH} used for the calculation was 1.14 × 10^3 M^{-1} hr^{-1} (23).

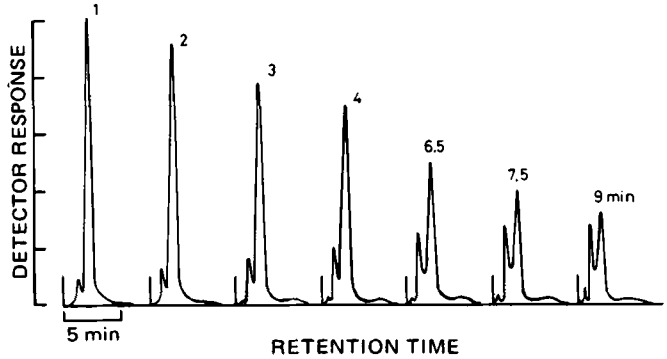


Figure 5—*HPLC changes for the cefazolin reaction with 0.10* M *ε-aminocaproic acid at pH 10.60, 35°, and 0.5 ionic strength with time in minutes (listed in graph).*

Table IV—Aminolysis Rate Constants of Cephalosporins and Penicillin G at 0.5 or 1.0 Ionic Strength (μ) and 35°

Amine	pKa[a]	Rate Constant[b]	β-Lactam Antibiotics: Cephalothin μ = 0.5	Cephalothin μ = 1.0[c]	Deacetylcephalothin μ = 0.5	Deacetylcephalothin μ = 1.0[c]	Cefazolin μ = 0.5	Cefazolin μ = 1.0[c]	Penicillin G μ = 0.5[a]	Penicillin G μ = 1.0[c]
ε-Aminocaproic acid	10.55	k_n	9.6	9.0	—	—[d]	9.1	9.6	5.3	24
		k_{gb}	873	1280	—	420	874	1380	1770	1100
		$k_{gOH} \times 10^{-4}$	12	12[e]	—	10	13	10[e]	10	16[e]
β-Alanine	9.98	k_n	2.0	3.0	—	1.2	3.4	3.0	1.0	6.0
		k_{gb}	109	258	—	114	122	270	213	240
		$k_{gOH} \times 10^{-4}$	3.5	3.2[e]	—	—[d]	2.1	3.2[e]	4.2	5.5
Glycine[f]	9.41	k_n	0.78	1.2	—[d]	—[d]	0.83	—[d]	0.40	3.6
		k_{gb}	73.6	150	27.7	48	81.5	162	90.6	96.0

[a] Determined previously (13). [b] Defined in Eq. 5; k_n in M^{-1} hr^{-1} and k_{gb} and k_{gOH} in M^{-2} hr^{-1}. [c] Values reported by Bundgaard (18). [d] Undetectable. [e] Recalculated from the reported values (18) according to the definition in Eq. 5 by use of the relationship (18) $\log[OH^-] = \log a_{OH^-} + 0.12$. [f] The k_{gOH} constant could not be determined.

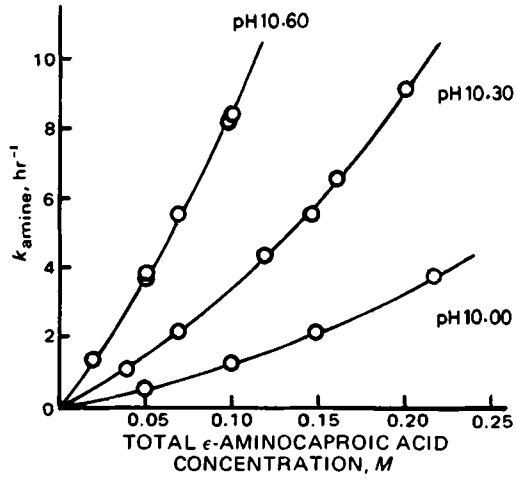


Figure 6—*Pseudo-first-order rate constants* versus *total amine concentration for cefazolin reaction with ε-aminocaproic acid at 35° and 0.5 ionic strength. Curves were calculated from Eq. 5 and rate constants in Table I.*

Aminolysis Kinetics—Typical plots of the aminolysis rate constant, k_{amine}, *versus* total amine concentration, $[amine]_T$ (Fig. 6) showed upward curvature at any pH. These pseudo-first-order rate constants apparently obey the general relationship reported previously for penicillins (7, 10, 13, 18) and cephalosporins (18):

$$k_{amine} = k_n[amine] + k_{gb}[amine]^2 + k_{gOH}[amine]a_{OH^-} \quad \text{(Eq. 5)}$$

where k_n represents the unassisted or water-catalyzed reaction of free amine on the β-lactam moiety, k_{gb} represents the general base-catalyzed and nucleophilic reaction with assistance by the amine second molecule, and k_{gOH} is the hydroxide ion-catalyzed third-order rate constant of nucleophilic displacement by the amine. The concentration of free amine can be calculated from:

$$[amine] = \frac{K_a}{K_a + a_{H^+}}[amine]_T \quad \text{(Eq. 6)}$$

where K_a is the conjugated amine acid dissociation constant. The pKa values determined previously under the same conditions (13) were used for the calculation.

Rearrangement of Eq. 5 leads to:

$$\frac{k_{amine}}{[amine]} = k_n + k_{gOH}a_{OH^-} + k_{gb}[amine] \quad \text{(Eq. 7)}$$

Equation 7 predicts that plots of $k_{amine}/[amine]$ *versus* [amine] provide k_{gb} as a slope of $k_n + k_{gb}a_{OH^-}$ as an intercept. One such plot is given in Fig. 7 for cephalothin, deacetylcephalothin, and cefazolin glycinolysis,

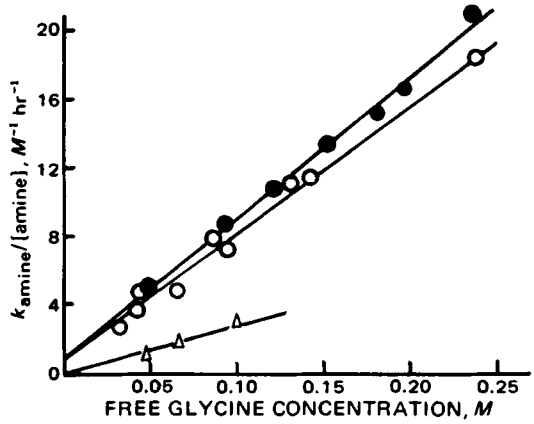


Figure 7—*Apparent second-order rate constants* versus *free amine concentration for the reactions of cephalothin (○), cefazolin (●), and deacetylcephalothin (△) with glycine at 35° and 0.5 ionic strength.*

Table V—Effect of Ionic Strength on the Reaction Rate Constants of Cephalothin with 0.12 *M* Glycine at pH 9.50 and 35°

Ionic Strength[a]	Rate Constant, hr^{-1}: k_{obs}[b]	k_{pH}[b,c]	k_{amine}[d]
0.5	0.506	0.207	0.299
1.0	0.649	0.213	0.436
2.0	0.772	0.217	0.555

[a] Adjusted with potassium chloride. [b] Determined by HPLC analysis. [c] $k_{pH} = (k_1)pH + (k_2)_{pH}$. [d] $k_{amine} = k_{obs} - k_{pH}$.

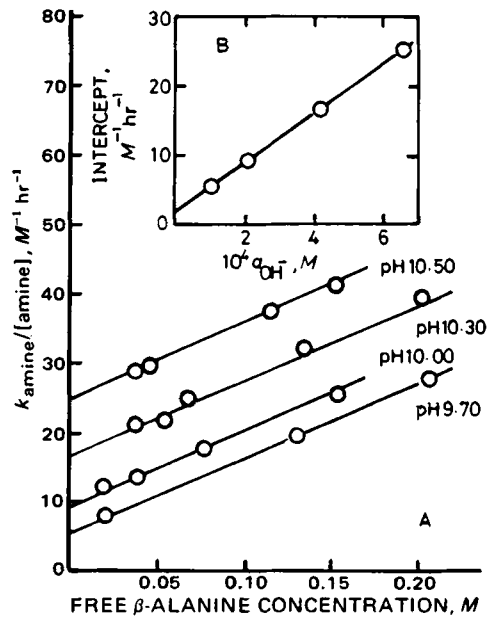
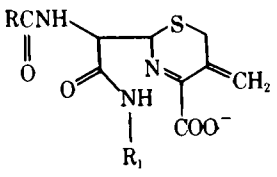
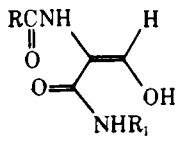


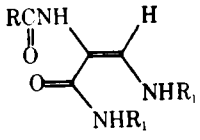
Figure 8—*Apparent second-order rate constants* versus *free amine concentration (A) and the intercepts of Fig. 8A* versus *hydroxide-ion activity (B) for cephalothin reaction with β-alanine at 35° and 0.5 ionic strength.*



I



II



III

showing a single line in each case with different slopes. Another typical plot with different intercepts and the same slope is illustrated in Fig. 8, predicting significant contribution of the kinetic term of k_{gOH}.

The weighed multiple linear regression analysis according to Eq. 7 provides (with $R > 0.98$) the rate constants for the uncatalyzed (or water-assisted), general base-catalyzed, and hydroxide ion-catalyzed reactions of cephalosporins with a number of amines (Table IV). The solid lines in Figs. 6–8 were calculated from the rate law (Eq. 5) and the parameters in Table IV and show satisfactory agreement with the data.

In cephalosporin aminolysis, the rate constants of k_{gb} were 50% lower at $\mu = 0.5$ than those determined spectrophotometrically (18) for similar aminolysis at 35° and $\mu = 1.0$ (Table IV). The UV spectrophotometric analysis (18) of the present reaction solutions gave rate constants almost identical to those determined by HPLC. This significant difference may be attributed to the different ionic strength rather than to the analytical method.

This speculation was substantiated in cephalothin glycinolysis at ionic strengths of 0.5–2.0 (Table V). The aminolysis rate constants, k_{amine}, compared to the hydrolysis rate constants, k_{pH}, increased significantly with the ionic strength increase. The ionic strength effect was similar to that for phenyl acetate aminolysis in which a high concentration of potassium chloride increased the reaction rates (27, 28).

General base-catalyzed cephalosporin aminolysis was favored by ionic strength increases and presumably proceeded through activated complexes of more ionic character than the starting materials. For the cephalosporin aminolysis (Table IV), the influence of ionic strength on the uncatalyzed (or water-assisted) and hydroxide-ion-catalyzed rate constants (k_n and k_{gOH}) was small compared to that on the amine-catalyzed rate constants (k_{gb}). In contrast to cephalosporin reactivity, the marked increase of k_n compared to k_{gb} and k_{gOH} by an increase of ionic strength was noted for penicillin aminolysis.

The rate constants for penicillin G aminolysis determined at $\mu = 0.5$ in this laboratory (13) and those determined previously at $\mu = 1.0$ (18) under the same temperature condition (35°) are also listed in Table IV. The rate constants k_n of penicillin G at $\mu = 0.5$ increased significantly five to nine times at $\mu = 1.0$, while the catalyzed rate constants were almost unchanged. These different reaction rate sensitivities to the ionic strength between pencillin and cephalosporin may result from the difference in the mechanism and/or ionic nature of the transition state.

There was no significant difference in cephalosporin aminolysis rate constants as obtained by HPLC and differential UV spectrometry. The HPLC method requires no assumption on the UV absorbability of the reaction products and permits simultaneous determination of the starting materials and degradation products. The UV data for the first three to five half-lives, when treated by literature methods (29), also provide reliable rate constants (18, 19).

Mechanism of Cephalosporin Aminolysis—The amine and hydroxide-ion-catalyzed reactions of cephalosporins are likely to be catalyzed nucleophilic amine displacements on the β-lactam moiety, yielding the corresponding cephalosporoylamide, as demonstrated for penicillin aminolysis (13, 16). The uncatalyzed or water-assisted kinetic term of Eq. 5 may represent a nucleophilic displacement reaction to yield the cephalosporoylamides or a kinetically equivalent general base-catalyzed hydrolysis to yield cephalosporoic acids. For penicillin aminolysis, this reaction was nucleophilic (13, 16). Cephalosporin reaction with one amine molecule represents nucleophilic displacement (18). Since the experimental evidence strongly suggested the dominance of the nucleophilic reaction, no further experiments were performed.

Relative Reactivity, Aminolysis Products, and Chemical Aspects of Cephalosporin Allergy—There are two striking differences between the chemistry of cephalosporins and penicillins. One is the relative rate of aminolysis. Change in the acylamide side chain in penicillin molecules produces no significant change in the aminolysis rate (7, 16, 30, 31), whereas the cephalosporin aminolysis rate is markedly influenced by the nature of the cephalosporin molecules.

In comparison with the reactivity of cephalosporins containing 7-position thienyl side chains, deacetylcephalothin is less reactive than cephalothin, as seen by the glycinolysis rates (Table IV). This difference can be attributed either to the inductive effect on the 3-methylene substituent (18) or to the leavability of the 3-methylene moiety in activating the β-lactam ring toward nucleophilic attack (32). As a result, cephalosporins containing a strong electron-withdrawing or good leaving group in the 3-position, such as cephaloridine, are readily destroyed in the presence of amines (18). Cefazolin and cephalothin reaction with amino acid primary amino groups resembled that of penicillin G (Table IV). These clinically used cephalosporins may conjugate with free protein amino groups in the body as readily as does penicillin G.

Furthermore, the penicillin β-lactam ring can be opened to give a relatively stable D-α-penicilloate without further molecular degradation. The presence of a Δ^3-double bond in cephalosporins leads to more extensive cleavage on hydrolysis and aminolysis (33, 34). The initial aminolysis product (I) with λ_{max} 230 nm was followed by chromophores with λ_{max} 270 and 278 nm (attributed to molecular fragmentation) and penaldates (II) and penamaldates (III) (33, 34). These actual structures are not completely understood. UV spectra of HPLC peaks, which appeared during cephalothin glycinolysis (Fig. 1B), revealed characteristic λ_{max} absorptions at 230 and 270 nm (Fig. 2). The UV spectrum of peak 5, which increased in peak height with time at the last aminolysis stage, exhibited λ_{max} at 278 nm. On the basis of reported chemical and spectroscopic evidence (33, 34) on the cephalosporin aminolysis product, peaks 3, 4, and 5 from the cephalothin glycinolysis may correspond to I, II, and III, respectively.

REFERENCES

(1) M. A. Schwartz, *J. Pharm. Sci.*, **58**, 643 (1969).

(2) M. W. Brandriss, J. W. Smith, and H. G. Steinmann, *J. Immunol.*, **94**, 696 (1965).

(3) F. R. Batchelor, J. M. Dewdney, R. D. Weston, and A. W. Wheeler, *Immunology*, **10**, 21 (1966).

(4) K. Shibata, T. Atsumi, Y. Horiuchi, and K. Mashimo, *Nature (London)*, **212**, 419 (1966).

(5) Y. Mine, M. Nishida, S. Goto, and S. Kuwahara, *J. Antibiot.*, **23**, 195 (1970).

(6) J. M. T. Hamilton-Miller and E. P. Abraham, *Biochem. J.*, **123**, 183 (1971).

(7) M. A. Schwartz and G.-M. Wu, *J. Pharm. Sci.*, **55**, 550 (1966).

(8) M. A. Schwartz, *ibid.*, **57**, 1209 (1968).

(9) C. H. Schneider and A. L. de Weck, *Biochim. Biophys. Acta*, **168**, 27 (1968).

(10) H. Bundgaard, *Dan. Tidsskr. Farm.*, **46**, 29 (1972).

(11) H. Bundgaard, *J. Pharm. Pharmacol.*, **24**, 985 (1972).

(12) T. Yamana, A. Tsuji, E. Miyamoto, and E. Kiya, *ibid.*, **27**, 283 (1975).

(13) A. Tsuji, T. Yamana, E. Miyamoto, and E. Kiya, *ibid.*, **27**, 580 (1975).

(14) T. Yamana, A. Tsuji, E. Miyamoto, and E. Kiya, *ibid.*, **27**, 771 (1975).

(15) H. Bundgaard, *ibid.*, **28**, 725 (1976).

(16) H. Bundgaard, *Arch. Pharm. Chemi, Sci. Ed.*, **4**, 91 (1976).

(17) H. Bundgaard, *Acta Pharm. Suec.*, **14**, 391 (1977).

(18) H. Bundgaard, *Arch. Pharm. Chemi, Sci. Ed.*, **3**, 94 (1975).

(19) H. Bundgaard, *Acta Pharm. Suec.*, **13**, 299 (1976).

(20) P. P. K. Ho, R. D. Towner, J. M. Indelicato, W. J. Wilham, W. A. Spitzer, and G. A. Koppel, *J. Antibiot.*, **26**, 313 (1973).

(21) J. M. Indelicato, T. T. Norvilas, R. R. Pfeiffer, W. J. Wheeler, and W. L. Wilham, *J. Med. Chem.*, **17**, 523 (1974).

(22) R. D. Kinget and M. A. Schwartz, *J. Pharm. Sci.*, **58**, 1102 (1969).

(23) T. Yamana and A. Tsuji, *ibid.*, **65**, 1563 (1976).

(24) J. Konecny, E. Felber, and J. Gruner, *J. Antibiot.*, **26**, 135 (1973).

(25) H. S. Harned and W. J. Hamer, *J. Am. Chem. Soc.*, **55**, 2194 (1933).

(26) E. Van Heyningen, *J. Med. Chem.*, **8**, 22 (1965).

(27) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, **82**, 675 (1960).

(28) W. P. Jencks and M. Gilchrist, *ibid.*, **88**, 104 (1966).

(29) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).
(30) H. Bundgaard, *Dan. Tidsskr. Farm.*, **46**, 85 (1972).
(31) T. Yamana, A. Tsuji, E. Kiya, and E. Miyamoto, *J. Pharm. Sci.*, **66**, 861 (1977).
(32) D. B. Boyd, R. B. Hermann, D. E. Presti, and M. M. Marsh, *J. Med. Chem.*, **18**, 408 (1975).
(33) J. M. T. Hamilton-Miller, G. G. F. Newton, and E. P. Abraham, *Biochem. J.*, **116**, 371 (1970).
(34) J. M. T. Hamilton-Miller, E. Richards, and E. P. Abraham, *ibid.*, **116**, 385 (1970).

ACKNOWLEDGMENTS

The authors acknowledge the gifts of cephalosporins from Shionogi & Co. and Fujisawa Pharmaceutical Co., Japan. They also thank Ms. A. Ito, Ms. T. Takamura, and Ms. K. Matsui for assistance.

Localization of Low Molecular Weight ^{99m}Tc-Labeled Dimercaptodicarboxylic Acids in Kidney Tissue

DAVID O. KUBIATOWICZ, THEODORE F. BOLLES, JAMES C. NORA, and DIONYSSIS S. ITHAKISSIOS ˣ

Received March 18, 1977, from the *Medical Products Division, 3M Co., 3M Center, St. Paul, MN 55101.* Accepted for publication October 27, 1978.

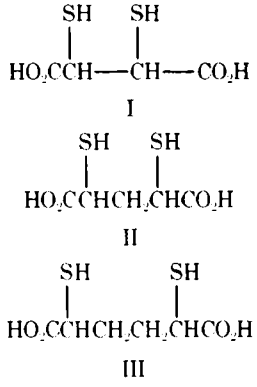
Abstract □ Kidney localization of low molecular weight ^{99m}Tc-dimercaptodicarboxylic acid complexes was examined in mice. The complexes ^{99m}Tc-dimercaptosuccinic acid, ^{99m}Tc-dimercaptoglutaric acid, and ^{99m}Tc-dimercaptoadipic acid were formed by reducing sodium ^{99m}Tc-pertechnetate with stannous chloride in the presence of 2–10-fold excess ligand at pH 2.5 or 7.5. Kidney specificity decreased as chain length between the mercapto groups increased. Optimum kidney retention occurred with complexes formed at pH 2.5. Complexes prepared at pH 7.5 were rapidly excreted through the urine and feces. Kidney localization of complexes prepared at one pH was not altered if the pH was later changed.

Keyphrases □ Technetium mercaptan complexes—localization in kidney tissue, synthesis, low molecular weight, structure–activity relationships □ Radionuclide imaging, renal—technetium mercaptan complexes, low molecular weight, localization in kidney tissue, synthesis, structure–activity relationships □ Kidney—radionuclide imaging, technetium mercaptan complexes, structure–activity relationships □ Structure–activity relationships—technetium mercaptan complexes, localization in kidney tissue

Radionuclide diagnosis of renal disease saves time in routine renal function tests and allows external kidney monitoring for clearance and morphological studies. The ^{203}Hg- and ^{197}Hg-labeled agents previously used have been replaced by ^{99m}Tc-labeled agents to decrease the high absorbed radiation dose *in vivo* (1).

Initial work on technetium mercaptan complexes as organ imaging agents led to the introduction of ^{99m}Tc-dihydrothioctic acid as a replacement for rose bengal in liver, gallbladder, and biliary tract studies (2). Other unpublished studies indicated that technetium complexes with short chain mono- and dimercaptodicarboxylic acids accumulated in kidney tissue. ^{99m}Tc-Dimercaptodicarboxylic acids were found to have better localization characteristics than their monomercapto analogs, especially ^{99m}Tc-dimercaptosuccinic acid, which has become well known as a renal imaging agent (1).

This *in vivo* study concerned organ localization of low molecular weight ^{99m}Tc-dimercaptodicarboxylic acids as affected by the number of carbon atoms between the mercapto groups and by the pH of complex formation.



I

II

III

EXPERIMENTAL

Dimercaptosuccinic acid[1] (I) was used without further purification.

Dimercaptoglutaric acid (II) was synthesized according to a literature procedure (3). Glutaric acid was brominated and esterified to form dimethyl 2,4-dibromoglutarate. The dibromide was reacted with potassium thioacetate to form the acetyl dithio ester. This derivative was subjected to alkaline hydrolysis in the presence of iodine and recrystallized from ethyl acetate to yield 1,2-dithiolane-3,5-dicarboxylic acid (the disulfide of II), mp 190–192° [lit. (3) mp 193–194°]. The structure for the disulfide was confirmed by elemental analysis, NMR, and mass spectrometry. Dimercaptoglutaric acid was generated from its disulfide, just prior to technetium complex formation, by reduction with aqueous sodium borohydride at pH 8, a technique that has been demonstrated in related compounds (4).

Dimercaptoadipic acid (III) was synthesized by incubating 2,2′-dibromoadipic acid[2] with aqueous potassium xanthogenate, followed by alkaline hydrolysis of the xanthate derivative in methanol and acidification. The solid was further purified by recrystallization from boiling water to yield pure crystalline III, mp 186–189° [lit. (5) mp 187–189°]. The structure for III was confirmed by elemental analysis and NMR.

^{99m}Tc-Dimercaptodicarboxylic Acid Complexes—These complexes were prepared in a nitrogen atmosphere by adding 0.1–2 ml of sodium pertechnetate ($Na^{99m}TcO_4$) (IV) eluate from ^{99}Mo-^{99m}Tc-generator[3] to 1–3 ml of stock tin–dimercaptodicarboxylic acid solution (pH 2.5 or 7.5), followed by 5–15 min of incubation at room temperature. The

[1] J. T. Baker, Phillipsburg, N.J.
[2] ICN Laboratories, Cleveland, Ohio.
[3] Mallinckrodt, St. Louis, Mo.

0022-3549/79/0500-0621$01.00/0