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Stability of D-5,5-Dimethyl-Δ^2-thiazoline-4-carboxylic Acid in Relation to Its Possible Occurrence As a Degradation Product of Penicillin by the Exocellular DD-Carboxypeptidase-Transpeptidase from *Streptomyces* R61 and the Membrane-bound DD-Carboxypeptidase from *Bacillus stearothermophilus**

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The stability of D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid has been studied under various conditions. In 10 mM cacodylate, pH 6.5, and at 55°C, D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (at concentrations lower than 1 mM) is hydrolyzed into *N*-formyl-D-penicillamine with a half-life of 3 to 4 min. On this basis, it is very unlikely that D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid could be one of the end products resulting from the cleavage of benzylpenicillin by the DD-carboxypeptidase of *Bacillus stearothermophilus* (as reported by Hammarström and Strominger (1976) *J. Biol. Chem.* 251, 7947–7949). In 3 mM phosphate, pH 7.5, and at 37°C, D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (at concentrations lower than 1 mM) has a half-life of 45 min. On the basis of kinetic experiments carried out under these conditions with phenoxymethylpenicillin and the DD-carboxypeptidase-transpeptidase of *Streptomyces* R61, it is concluded that the primary product which arises from the thiazolidine moiety of the antibiotic molecule and gives rise to *N*-formyl-D-penicillamine, has a half-life of 10 min, a value which is not compatible with the hypothesis that D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid would be an intermediate involved in the fragmentation pathway.

DD-Carboxypeptidases-transpeptidases are constituents of the peptide cross-linking enzyme system involved in the synthesis of the bacterial wall peptidoglycan. They react with β-lactam antibiotics (penicillins and cephalosporins) to form inactive complexes which, in turn, slowly break down with the result that the enzyme is regenerated whereas the antibiotic is released in the form of inactive metabolites (1, 2). The following model has been proposed for the interaction between penicillin (I) and the exocellular DD-carboxypeptidase-transpeptidase (E) excreted by *Streptomyces* R61 (in short the R61 enzyme): $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E$ + degradation products (EI^*, inactive stoichiometric complex in which both the enzyme and the inhibitor are modified; K, dissociation constant; k_3 and k_4, first order rate constants). By using radioactive benzylpenicillins ^{14}C-labeled on the acyl side chain and ^3H-labeled on the β-methyl group of the thiazolidine moiety, respectively, it was shown (3, 4) that during breakdown of the complex EI^* formed with the R61 enzyme and within the limits of experimental error, release of the ^{14}C label, release of the ^3H label and enzyme reactivation were all concomitant events and that the end products of the reaction were [^{14}C]phenylacetylglycine and *N*-[^3H]formyl-D-penicillamine. The membrane-bound DD-carboxypeptidase of *Bacillus stearothermophilus* also fragments benzylpenicillin with formation of phenylacetylglycine (5). However, Hammarström and Strominger claimed that 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid, and not *N*-formyl-D-penicillamine, was the fragment arising from the thiazolidine moiety of the antibiotic molecule (6). Δ^2-Thiazoline-4-carboxylic acid and its 2-methyl derivative are known to be stable only at extreme pH values whereas they are rapidly hydrolyzed in dilute acids and in neutral solutions (7–9). Since different substituents have a marked influence on the hydrolysis of Δ^2-thiazolines (9), it was felt necessary to study the stability of 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid under various conditions and more especially the sensitivity to hydrolysis that this thiazoline exhibits in neutral solutions.

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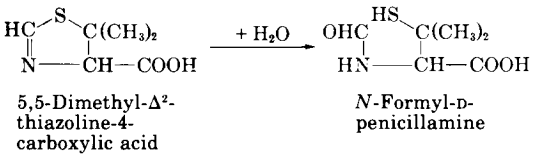


5,5-Dimethyl-Δ^2-thiazoline-4-carboxylic acid

N-Formyl-D-penicillamine

The information thus obtained was then used for a critical re-examination of the enzymatic cleavage of penicillin by both the R61 and *B. stearothermophilus* enzymes.

MATERIALS AND METHODS

Physical Methods – Melting points were determined with a Büchi-Tottoli apparatus. Optical rotations were measured on a Thorn-NPL photoelectric polarimeter, type 243. UV spectra were recorded with either a Beckman spectrophotometer or a Zeiss spectrophotometer model M4QIII. IR spectra (KBr discs) were run on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained with either a Hitachi Perkin-Elmer spectrometer, model R-24, or a WH270 Bruker spectrometer. Mass spectra were recorded on a AEI MS 12 apparatus at 8 kV accelerating potential, 100 μA anode current, and 70 eV ionization energy. Circular dichroism measurements were made with a Cary 61 spectropolarimeter. The cell compartment was continually purged with dry, purified, nitrogen. A time constant of 10 s, a scanning speed of 12 nm/min, quartz cells of 0.2 cm or 1.0 cm pathlength and a concentration of 0.1 mg/ml were used. Gas-liquid chromatography (GLC) was carried out at 125°C on a Pye 104 gas chromatograph with a column (150 cm × 4 mm inside diameter) of 3% OV-17 on Gas-chrom Q (100 to 120 mesh); the carrier gas was nitrogen at a flow rate of 60 ml/min. High voltage paper electrophoresis was carried out on Whatman No. 3MM (150 × 4 cm) paper at pH 3.5 (pyridine:acetic acid:water, 1:10:89) and at pH 8.2 (1 % NH_4 bicarbonate buffer adjusted to pH 8.2 with NaOH). Radioactive spots were localized by scanning with a Packard radiochromatogram scanner, model 7200. Radioactive samples were mixed with Lumagel (Lumac) and counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3390, with absolute activity analyzer, model 544.

Titration of Free Sulfhydryl Groups – Samples (5 to 260 μl) containing free —SH groups were supplemented at 37°C with 30 μl of 1 M sodium phosphate buffer, pH 7.0, and, when necessary the total volume was adjusted to 290 μl with water. After the addition of 10 μl of a 50 mM solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.) in ethanol, the samples were mixed by stirring and then transferred into 500-μl microcells for absorbance measurements at 420 nm, using a Zeiss spectrophotometer with the cell compartment maintained at 37°C. With pure *N*-formyl-D-penicillamine, and in the above 0.1 M phosphate buffer, pH 7.0, the reaction was 95% complete within 60 s. After reaction of a 0.1 mM solution of *N*-formyl-D-penicillamine with DTNB,[1] the resulting solution had an absorbance of 1.15.

Enzymes – The exocellular enzyme from *Streptomyces* R61 was purified to protein homogeneity (10). The DD-carboxypeptidase activity was estimated by incubating the enzyme with 100 nmol of Ac_2-L-Lys-D-Ala-D-Ala in 30 μl, total volume, of 3 mM sodium phosphate, pH 7.0 (for more details, see Ref. 10). The membrane-bound DD-carboxypeptidase of *B. stearothermophilus* was a gift from Dr. P. E. Reynolds, University of Cambridge, U. K.

N-Formyl-D-penicillamine – A cooled (0°C) solution of D-penicillamine·HCl (557 mg, 3 mmol) in 95% formic acid (7 ml) containing sodium formate (200 mg, 3 mmol) was supplemented, dropwise, with acetic anhydride (2 ml), over a period of 15 min. The solution was kept for 1 h at room temperature and then was further supplemented with 3 ml of ice water. The mixture was evaporated to dryness under reduced pressure at a bath temperature of 30°C. The residue was taken up in 50 ml of water and the solution was evaporated to dryness as above. This operation was repeated twice. The residue was crystallized from 3 ml of boiling water, yielding 500 mg of the title compound, m.p. 140–145°C. Recrystallization from boiling water yielded 300 mg (1.69 mmol, 56% yield) pure *N*-formyl-D-penicillamine, m.p. 146–148°C. $[\alpha]_D^{25}$ +63 (*c* = 1, pyridine), +23 (*c* = 2, 1 M phosphate, pH 7.0), +13 (*c* = 2.3, NaOH 2 N); IR ν_{max} 3290 (NH), 1700 (COOH), 1620 (amide I), 1530 (amide II), 1380 and 1290 cm^{-1}; NMR (45 mg dissolved in 0.5 ml of 0.5 N NaOD in D_2O, pH about 12) δ (in parts per million relative to HOD signal; peaks upfield with respect to HOD-peak have negative δ values) −3.37 and −3.27 (*s*, $C(CH_3)_2$), −0.77 (*d*, J = 1 Hz, CHCOONa), +3.33 (*d*, J = 1 Hz, N—CHO). An $[\alpha]_D$ value of +63 (pyridine) but no melting point has been described (11). It should be noted that the conditions of formylation are milder than those described (12) where a rather extensive racemization was observed. Even in our procedure, recrystallization was necessary to obtain a pure enantiomer.

D-5,5-Dimethyl-Δ^2-thiazoline-4-carboxylic Acid Hydrochloride – A suspension of *N*-formyl-D-penicillamine (1.2 g, 6.77 mmol) in 60 ml of dry ether was saturated with hydrogen chloride at 0°C and allowed to stand overnight. The crystalline precipitate of the hydrochloride (1 g, 5.11 mmol, 75% yield) was collected, m.p. 138–140° (decomposition). Recrystallization from absolute ethanol-ether raises the m.p. to 145–146°C (decomposition). $[\alpha]_D^{25}$ −47 (*c* = 2, NaOH 2 N). IR ν_{max} 3000 to 2500 (NH^+), 1725 (COOH), 1585 (C=N), 1390, 1240, 1210, and 1200 cm^{-1}; NMR (45 mg in 0.5 ml of 1 N NaOD in D_2O, pH about 12) δ (relative to HOD signal) −3.32 and −3.07 (*s*, $C(CH_3)_2$), −0.30 (*d*, J = 2.3Hz, CHCOONa), +3.47 (*d*, J = 2.3 Hz, SCH=N). A melting point of 162–163°C (decomposition) has been described (13).

N-[^{3}H]Formyl-D-penicillamine and [2-^{3}H]D-5,5-Dimethyl-Δ^2-thiazoline-4-carboxylic Acid Hydrochloride – Tritiated *N*-formyl-D-penicillamine was prepared from 300 mg of D-penicillamine (2 mmol), 1.5 ml of 98 to 100% formic acid (*d* = 1.22, 40 mmol) containing 10 mCi of ^{3}H-COONa (The Radiochemical Centre, Amersham, England) and 0.6 ml of acetic anhydride and further isolated as described for the nonradioactive compound. Yield 230 mg = 1.3 mmol = 65%. Part of the product was diluted with an equal amount of nonradioactive *N*-formyl-D-penicillamine and recrystallized from boiling water; specific activity: 124 μCi/mmol. Tritiated D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid HCl was prepared from *N*-[^{3}H]formyl-D-penicillamine (130 mg, nonrecrystallized) suspended in 6 ml of dry ether and saturated with hydrogen chloride at 0°C. The precipitate (85 mg, specific activity 253 μCi/mmol) was diluted with 70 mg of thiazoline·HCl and purified by crystallization from absolute ethanol-ether. Yield 128 mg; specific activity 125 μCi/mmol.

Reaction of *N*-formyl-D-penicillamine with bis(trimethylsilyl) acetamide in acetonitrile at room temperature for 15 min, gave one product with a retention time of 18 min on GLC. GLC-mass spectrometry showed that it was the Tris-trimethylsilyl (Me_3Si) derivative (M^+ 393). The 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid hydrochloride gave with the same reagent a mono Me_3Si ester (M^+ 231) with a retention time of 3 min. The purity of the radioactive product was checked by this method.

Tritiated Di(*N*-formyl-D-penicillamine)disulfide was prepared by oxidizing *N*-[^{3}H]formyl-D-penicillamine with ferric chloride (4).

N-Formyl-penicillamine (Potassium Salt) and D-5,5-Dimethyl-Δ^2-thiazoline-4-carboxylate (Potassium Salt) – The potassium salt of *N*-formyl-penicillamine was prepared by dissolving *N*-formyl-D-penicillamine in methanol and adding a slight excess of 2 M potassium-2-ethylhexanoate in acetone. It was crystallized by addition of ethyl acetate. The structure was confirmed by NMR.

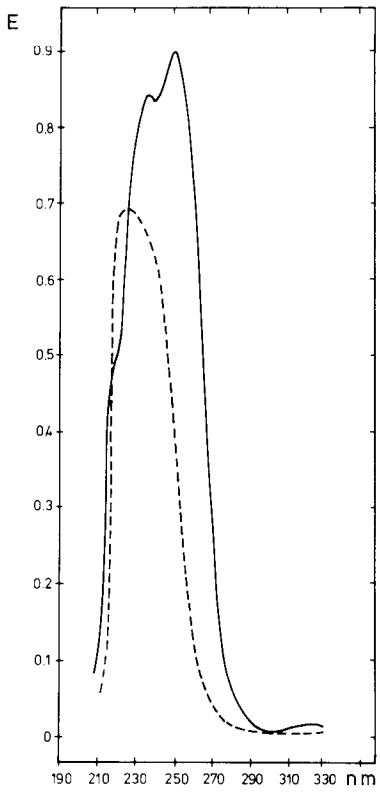
The potassium salt of the thiazoline was prepared by dissolving 1 eq of thiazoline-HCl in 2 eq of 1 N KOH. After evaporation to dryness under vacuum at room temperature, the potassium thiazoline was separated from KCl by extraction with methanol. The methanol solution was evaporated to dryness and the residue triturated with ether. The structure was also confirmed by NMR.

RESULTS

Stability of 5,5-Dimethyl-Δ^2-thiazoline-4-carboxylate in 0.1 N NaOH – Fig. 1 gives the UV spectra in 0.1 N NaOH and at 20°C of both *N*-formyl-D-penicillamine (with a maximum at 224 nm) and 5,5-dimethyl-Δ^2-thiazoline-4-carboxylate (with maxima at 254 and 236 nm). In this medium, the $[\alpha]_D$ value and both the UV and NMR spectra of the thiazoline compound remained unmodified for at least 5 h at 20°C, indicating that hydrolysis did not occur and therefore that the alkaline hydrolysis of the methyl ester (6) is a valid procedure for its synthesis.

Stability of 5,5-Dimethyl-Δ^2-thiazoline-4-carboxylic Acid and N-Formyl-D-penicillamine in Acidic Solutions – In 0.1 N HCl and at 20°C, a freshly made thiazoline solution (0.25 mg/

[1] The abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).



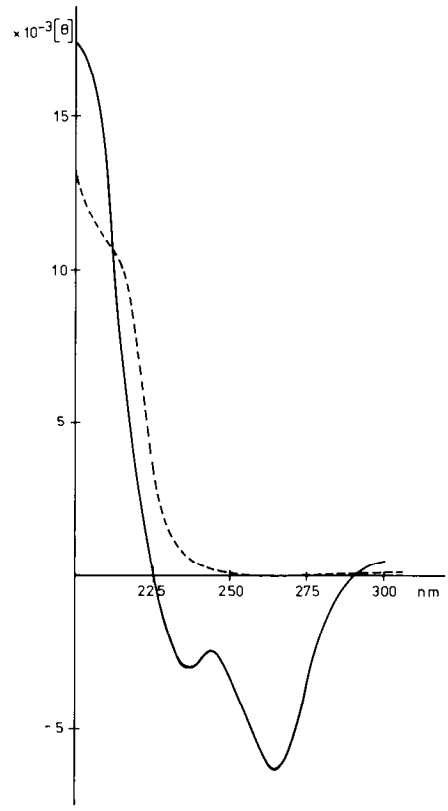


FIG. 1 (*left*). UV spectrum of D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid hydrochloride (1.6 mg; ——) and *N*-formyl-D-penicillamine (0.5 mg; - - -) in 0.1 N NaOH (20.0 ml) and at 20°C.

FIG. 2 (*right*). Circular dichroism spectrum of D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid hydrochloride (——) and *n*-formyl-D-penicillamine (- - -) in 10 mM sodium cacodylate, pH 7.0, at 20°C (0.1 mg/ml).

ml) had UV maxima at 229 and 265 nm. As the time increased, the extinction at 265 nm decreased progressively until a minimal value was reached after about 18 min. Conversely, *N*-formyl-D-penicillamine (0.25 mg/ml) showed, in addition to a peak at 224 nm, a maximum at 265 nm which increased in intensity with time until a maximal value was also reached after about 18 min. Hence, in 0.1 N HCl, the two compounds underwent interconversion and at the equilibrium position, *N*-formyl-D-penicillamine and the thiazoline occurred in a molar ratio of about 5.5 to 1. It should be mentioned that ring closure of *N*-formyl- and *N*-acetylcysteine in strong acids has been demonstrated (7, 8) and that the same reaction has also been observed with more complex molecules like glutathione (14, 15).

The $[\alpha]_D$ value of a 2% solution of 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid HCl in water (pH 1.4) at 25°C increased from +2.6 after 1 min to +8.5 after 7 min and then remained unchanged. From the solution thus obtained, a crystalline material precipitated slowly which was identified as *N*-formyl-D-penicillamine by silylation and GLC. Similarly, [2-^{3}H]5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (1.65 mg) was dissolved in 4 ml of a saturated solution of $(NH_4)_2SO_4$ (acidified with H_2SO_4 to pH 2.0) and extracted four times with 2 ml of ethyl acetate. The product recovered in the organic solvent (99% of the initial radioactivity) was characterized as *N*-formyl-D-penicillamine by silylation and GLC.

Mass Spectrometry—Both *N*-formyl-D-penicillamine and 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid gave rise to several identical fragments (Table I) and therefore, these data, as well as those published previously (6), must be interpreted with caution. Not all the peaks shown in Table I corresponded to those described by Hammarström and Strominger (6) for the thiazoline compound (that they probably obtained from the alkali salt) but the experimental conditions they used were not reported in sufficient detail and the relative intensity of the peaks was not mentioned.

Stability of 5,5-Dimethyl-Δ^2-thiazoline-4 Carboxylate in Neutral Solutions and in Solvent Pyridine:acetic acid:water of pH 3.5—Fig. 2 shows the circular dichroism spectra of 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid and *N*-formyl-D-penicillamine. The hydrolysis of the thiazoline into *N*-formyl-D-penicillamine was followed by measuring the ellipticity at 265 nm, by optical rotation at 589 nm, by reaction with DTNB and by NMR, respectively. In all cases, hydrolysis of the thiazoline reached completion. At concentrations lower than 1 mM, the hydrolysis was found to be a pseudo-first order reaction. At higher concentrations, the half-life of the thiazoline was concentration-dependent, the higher the concentration the shorter the half-life values (Table II, Experiments 1 to 6). The buffer composition (Experiments 7 to 9) and the temperature (Experiments 10 to 11) were also important parameters. Experiments 11 to 16, all carried out at thiazoline concentrations smaller than 1 mM, were especially relevant to the present investigations. 1) Experiments 11 and 12. These conditions are those used by Hammarström and Strominger (6) for the breakdown of the complex formed between benzylpenicillin and the *B. stearothermophilus* enzyme. The half-life value (about 3 to 4 min) was not modified in the presence of isolated membranes of *B. stearothermophilus* at concentrations of 10 to 40 mg of protein/ml. In the presence of membranes, however, —SH groups progressively disappeared from the reaction mixture probably as a result of the oxidation of *N*-formyl-D-penicillamine into di(*N*-formyl-D-penicillamine) disulfide. Previous experiments (4) suggested that the R61 enzyme might also somehow participate in the oxidation of the released *N*-formyl-D-penicillamine. 2) Experiment 13. In 0.1 M

TABLE I

Mass spectra of N-formyl-D-penicillamine and 5,5-dimethyl-Δ²-thiazoline-4-carboxylic acid

N-Formyl-D-penicillamine					5,5-Dimethyl-Δ²-thiazoline-4-carboxylic acid HCl		
m/e	Fragment	Relative intensity at 170°C	150°C	135°C	*m/e*	Fragment	Relative intensity at 120°C
178	$M + 1$	0.2					
177	M^+	Trace					
160	$M + 1 - H_2O$ (m^* 143.8)	0.5	0.4				
159	$M - H_2O$	1.3	1.2		159	M^+	34.3
144	$M - SH$ $159 - CH_3$	0.3	0.3		144	$M - CH_3$ (m^* 130.4)	2.3
132	$M - COOH$	0.3	0.4				
126	$159 - SH$ $144 - H_2O$ (m^* 110.2)	1.9	2.1	2.3	126	$M - SH$	8.4
115	$159 - CO_2$ $159 - CS$	1.1	1.0		115	$M - CO_2$ $M - CS$	16.2
114	$159 - COOH$ $159 - CHS$	3.3	3.2	3.5	114	$M - COOH$ $M - CHS$	54.1
103	[HOOC—CH—NHCHO⁺]*	4.0	4.4	5.3			
100	$115 - CH_3$ (m^* 87.0) $159 - NCHS$	6.3	5.6	2.0	100	$115 - CH_3$ (m^* 87.0) $M - NCHS$	100
98	$132 - H_2S$ (m^* 72.8)	4.3	5.2	5.9			
87	$114 - HCN$ (m^* 66.4)	4.7	4.0	4.1	87	$114 - HCN$ (m^* 66.4)	31.9
85	$103 - H_2O$ (m^* 70.1)	9.0	10.0	10.6			
75	$(CH_3)_2CSH^+$	100	100	100	75	$(CH_3)_2CSH^+$	73.0

TABLE II

Half-life values of D-5,5-dimethyl-Δ²-thiazoline-4-carboxylic acid

In all cases, the hydrolysis of the thiazoline compound into *N*-formyl-D-penicillamine reached completion.

Experiments	Buffer	Final pH	Thiazoline concentration	Temperature	Half-life	Method utilized[a]
			mM		*min*	
1	50 mM phosphate, pH 7.0		10	20°C	25	NMR[b]
2	50 mM phosphate, pH 7.0		5	20	45	NMR
3	50 mM phosphate, pH 7.0		2.5	20	75	NMR
4	50 mM phosphate, pH 7.0		1.25	20	90	NMR
5[c]	50 mM phosphate, pH 7.0		0.5–1	20	110 (± 10)	NMR
6[d]	10 mM phosphate, pH 7.0	7.0	0.01–1	37	25 (± 4)	DTNB
7	1 M phosphate, pH 7.0	6.6	100	25	5	OR
8	1 M cacodylate, pH 7.0	6.9	100	25	7	OR
9[e]	80 mM cacodylate, pH 7.0	6.6	100	25	36	OR
10	10 mM cacodylate, pH 7.0	6.6	0.5	40	9	CD
11[f]	10 mM cacodylate, pH 7.0	6.6	0.5	55	4	CD
12	10 mM cacodylate, pH 6.5	6.5	0.6	55	2.5	DTNB
13	100 mM phosphate, pH 7.0	7.0	0.02	37	8	DTNB
14	3 mM phosphate, pH 7.5	7.5	0.010–0.025	37	45 (± 5)	DTNB
15	Pyridine:acetic acid:water, pH 3.5		0.6	0	1.5	DTNB
16	Pyridine:acetic acid:water, pH 3.5		0.6	22	<1	DTNB

[a] The abbreviations used are: NMR, nuclear magnetic resonance; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); OR, optical rotation; CD, circular dichroism.

[b] NMR: the half-life values were determined by measuring the integrated areas of the four lines characteristic of D-5,5-dimethyl-Δ²-thiazoline-4-carboxylic acid (8.16, 4.46, 1.60, and 1.38 ppm) and of its hydrolysis product *N*-formyl-D-penicillamine (8.06, 4.28, 1.45, and 1.38 ppm). The parts per million values reported here are relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The 1.60 and 1.38 ppm lines of thiazoline and the 1.45 and 1.38 ppm lines of *N*-formyl-D-penicillamine are the methyl lines.

[c] Experiment 5: the thiazoline concentrations used were 0.52, 0.66, 0.78, and 1 mM, respectively.

[d] Experiment 6: 13 different thiazoline concentrations, ranging from 10 μM to 1 mM, were used.

[e] Experiment 9: the sample also contained 1 eq of NaOH.

[f] Experiment 11: the half-life value of 4 min is an estimate because it was difficult to obtain an equilibrium between the temperature of the solution and that of the cuvette within the short time period needed for the reaction.

phosphate, pH 7.0, and at 37°C, the reaction between DTNB and *N*-formyl-D-penicillamine is virtually complete in 1 min (see "Materials and Methods"). Hence, less than 10% of the thiazoline initially present in a mixture of thiazoline and *N*-formyl-D-penicillamine is hydrolyzed during the time needed for the measurement of the free —SH groups. 3) Experiment 14. These conditions are those used for the breakdown of the complex formed between phenoxymethylpenicillin and the R61 enzyme (see text below). The presence of the R61 enzyme in the reaction mixture did not alter the half-life value. 4) Experiments 15 and 16. This solvent (see "Materials and Methods") in which the thiazoline has a half-life of about 1 min is that used by Hammarström and Strominger (6) for the separation of 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid by paper electrophoresis (precise temperature not mentioned). It was therefore not surprising to observe that with this solvent, [2-^{3}H]5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid had the same electrophoretic migration as *N*-[^{3}H]formyl-D-penicillamine (about 15 cm/h at 60 V/cm, toward the anode). Moreover, tritiated di(*N*-formyl-D-penicillamine) disulfide was also undistinguishable from *N*-[^{3}H]formyl-D-penicillamine under these conditions.

Crystallization Studies of N-Formyl-D-penicillamine (Potassium Salt) and D-5,5-Dimethyl-Δ^2-thiazoline-4-carboxylate (Potassium Salt) — Hammarström and Strominger (6) reported that the released ^{35}S compound arising from [^{35}S]benzylpenicillin co-crystallized with potassium thiazoline by using the system methanol:ethyl acetate (1:9, v/v). In this respect, the following experiments were carried out.

1. Thiazoline (potassium salt: 1 g) was dissolved in 2 ml of methanol. Addition of 20 ml of ethyl acetate only gave rise to about 10 mg of crystalline material. It was not further characterized.

2. *N*-Formyl-D-penicillamine (potassium salt: 100 mg) also dissolved in 2 ml of methanol readily crystallized under the above conditions. The yield was 90%.

3. Equimolar amounts (500 μmol) of *N*-[^{3}H]formyl-penicillamine (potassium salt; specific radioactivity: 6.74 μCi/mmol) and nonradioactive thiazoline (potassium salt) were dissolved in 2 ml of methanol. Upon addition of 20 ml of ethyl acetate, the specific radioactivity of the crystalline material thus obtained represented 83% of that of the original *N*-formyl-D-penicillamine. This material was recrystallized to constant specific radioactivity (90%). This value suggested that the thiazoline potassium salt preparation contained about 10% of *N*-formyl-penicillamine.

4. In this last experiment which resembled that described by Hammarström and Strominger (6), *N*-[^{3}H]formyl-D-penicillamine (5 mg; *i.e.* 28 μmol, specific radioactivity: 120.3 μCi/mmol) and 148 mg (*i.e.* 752 μmol) of nonradioactive thiazoline (potassium salt) were dissolved in 0.5 ml of methanol. Upon addition of 4.5 ml of ethyl acetate, 24 mg of crystalline material was obtained (chemical yield: 15.7%) which contained 61% of the total radioactivity of the initial mixture. Two subsequent crystallizations gave rise to crystals exhibiting constant radioactivity (17 μCi/mmol) with high chemical yields (70 and 78%, respectively). These data indicated that the radioactive *N*-formyl-penicillamine co-crystallized with some nonradioactive *N*-formyl-penicillamine present as an impurity (about 15 to 20%) in the thiazoline potassium salt preparation.

It should be emphasized that, in their crystallization experiments, Hammarström and Strominger (6) obtained similar results, *i.e.* a high radiochemical yield and a low chemical yield in the first crystallization, followed by a high chemical yield and a constant specific activity in the second crystallization.

Breakdown of R61 Enzyme·Phenoxymethylpenicillin Complex. Enzyme Reactivation and Release of Free —SH Groups — In order to check whether 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid might be the primary product which arises from the thiazolidine moiety of penicillin by interaction with the R61 enzyme and, in turn, gives rise to *N*-formyl-D-penicillamine by simple hydrolysis, it was important to find experimental conditions where the half-life of the complex formed between the enzyme and the selected β-lactam antibiotic was short when compared with that of the thiazoline compound. For this purpose, phenoxymethylpenicillin was used instead of benzylpenicillin because of the shorter half-life exhibited by the phenoxymethylpenicillin·R61 enzyme complex (1, 2). At 37°C and in 3 mM phosphate, pH 7.5, this latter *EI** complex had a half-life of 38 min, which corresponds to a k_4 value (see the introduction) of 3.1×10^{-4} s^{-1} (a 2.8×10^{-4} s^{-1} value had been found previously) (1, 2). Under the same conditions and at a 25 μM concentration, 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid had a half-life of about 45 ($\pm$5) min (Table II). Breakdown of the complex, at an initial 33 μM concentration, was studied by measuring both the enzyme reactivation and the release of the free —SH groups as a function of time. The time course experiment (Fig. 3) showed that enzyme reactivation (○——○) and appearance of free —SH groups (●——●) were not concomitant. Hence, *N*-formyl-

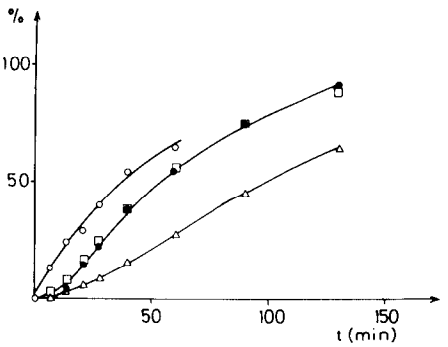


FIG. 3. Breakdown of the complex formed between phenoxymethylpenicillin and the R61 enzyme. Time course of enzyme reactivation (○——○) and release of free —SH groups (●——●). The stock enzyme solution was dialyzed against 3 mM phosphate, pH 7.5, and the protein concentration adjusted to 1.4 mg/ml (final concentration: 38 μM) with the same buffer. Samples (250 μl) containing 9.5 nmol of enzyme were supplemented with 20 μl of the same phosphate buffer containing 400 nmol of phenoxymethylpenicillin, incubated at room temperature for 5 min (complex formation), supplemented with 20 μl containing 8 IU of β-lactamase (Riker) and finally incubated at 37°C (destruction of the excess of phenoxymethylpenicillin and breakdown of the complex enzyme·antibiotic). The initial complex concentration was 33 μM. After increasing times, 5-μl samples were removed from the reaction mixtures, diluted 21-fold and the resulting solutions used for the estimation of the enzyme activity (by incubating 5 μl of the diluted solutions with 100 nmol of Ac_2-L-Lys-D-Ala-D-Ala in 30 μl of 3 mM phosphate, pH 7.0, for 5 min at 37°C). The remainders of the reaction mixtures were used for the estimation of free —SH groups. They were supplemented with 30 μl of 1 M phosphate, pH 7.0, and 10 μl of 50 mM DTNB, the resulting solutions stirred and the absorbances measured at 420 nm after 1 min. The figure also shows the time courses of the release of the free —SH groups assuming the transitory formation of an intermediate compound with a half-life of 10 min (□——□) and 45 min (△——△), respectively. Under the present experimental conditions, a half-life of 45 min is that exhibited by 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (at a 25 μM concentration) (Table II).

D-penicillamine was not the primary product arising from the thiazolidine moiety of phenoxymethylpenicillin during breakdown of the enzyme·antibiotic complex, but its release must proceed via the transitory formation of an intermediate compound Z : $EI^* \xrightarrow{k_4} E + Z \xrightarrow{k_5} E +$ *N*-formyl-D-penicillamine. The velocity of the reaction is therefore given by

$$\frac{[N\text{-formyl-D-penicillamine}]}{[EI^*]_0} = 1 + \frac{k_4 e^{-k_5 t} - k_5 e^{-k_4 t}}{k_5 - k_4}$$

On the basis of this model and of a k_4 value of 3.1×10^{-4} s^{-1}, a theoretical curve (□——□) was calculated which coincided exactly with the experimental one providing that the intermediate Z had a half-life of 10 min (which corresponds to a k_5 value of 11.6×10^{-4} s^{-1}). An intermediate Z having the same 45-min half-life as 5,5-dimethyl-Δ^2-thiazoline-4-carboxylate would give rise to a curve (△——△) which drastically differs from the experimental one.

DISCUSSION

Hammarström and Strominger (6) proposed that the product arising from the thiazolidine ring of benzylpenicillin as a consequence of its fragmentation by the membrane-bound DD-carboxypeptidase of *B. stearothermophilus* was 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid. In their experiments, breakdown of the complex formed between [^{35}S]benzylpenicillin and the isolated membranes was carried out in 10 mM sodium cacodylate buffer, pH 6.5, and 55°C for 50 min in the presence of 0.6 mM nonradioactive standard thiazoline, *i.e.* under conditions where the thiazoline has a half-life of 3 to 4 min. Moreover, the released ^{35}S-labeled compound was seemingly characterized by paper electrophoresis at pH 3.5 (also in the presence of nonradioactive standard thiazoline), *i.e.* under conditions where the thiazoline has a half-life of 1 to 2 min (depending upon the temperature). Neither the nonradioactive thiazoline nor the ^{35}S-labeled thiazoline, assuming that this latter compound was really formed during the reaction, could survive the above treatments. It should also be noted that on the basis of our own data, the co-crystallization experiments reported by Hammarström and Strominger (6) are the best explained by assuming the presence of a large amount of *N*-formyl-D-penicillamine in their potassium thiazoline preparation. Finally, on the basis of radioactivity measurements, [^{3}H]thiazoline and *N*-[^{3}H]formyl-D-penicillamine had the same migration (16 cm) toward the anode by paper electrophoresis (60 min; 30 V/cm; cooled tank) at pH 8.2 in bicarbonate buffer (under which conditions, the thiazoline should be reasonably stable). Hence it seems very likely that the released ^{35}S-labeled product was mischaracterized. Consequently, the mechanism proposed for the enzymatic fragmentation of penicillin on the assumption that 5,5-dimethyl-Δ^2-thiazoline carboxylic acid was one of the primary products released, remains entirely hypothetical.

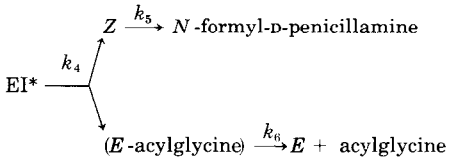
Experiments carried out with phenoxymethylpenicillin and the R61 enzyme show that the primary product which arises from the thiazolidine moiety of the antibiotic molecule and, in turn, gives rise to *N*-formyl-D-penicillamine, has a half-life of 10 min in 3 mM phosphate buffer, pH 7.5, and at 37°C. It is very unlikely that 5,5-dimethyl-Δ^2-thiazoline-4-carboxylate, which has a half-life of 45 min under these conditions, would be the intermediate formed during the reaction. The nature of this intermediate (Z) remains to be discovered. In particular, it is not known whether Z is a fragment of the penicillin molecule or, conversely, is a degraded but not fragmented penicillin metabolite. Depending upon the cases, two pathways may be proposed for the breakdown of the complex EI^* formed between the enzyme and penicillin:

$$EI^* \xrightarrow{k_4} E + \text{acylglycine} + Z \xrightarrow{k_5} E + \text{acylglycine} + N\text{-formyl-D-penicillamine}$$

or

$$EI^* \xrightarrow{k_4} E + Z \xrightarrow{k_5} E + \text{acylglycine} + N\text{-formyl-D-penicillamine}$$

A third possibility may also exist:



which is compatible with the experimental data providing that the breakdown of the enzyme·acylglycine complex is a rapid process. A precise determination of the kinetics of release of the acylglycine fragment should help in making a choice between the various alternatives.

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