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Further Degradation Products of Cephalosporin C

ISOLATION AND SYNTHESIS OF 2-(4-AMINO-4-CARBOXYBUTYL)THIAZOLE-4-CARBOXYLIC ACID

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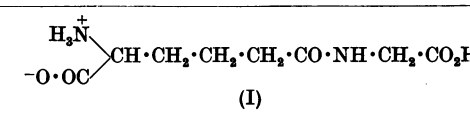
Abraham & Newton (1956) showed that cephalosporin C closely resembled (D-4-amino-4-carboxybutyl)penicillin (cephalosporin N) in some of its chemical properties but that it differed from a penicillin in others. On hydrogenolysis with Raney nickel and subsequent hydrolysis with acid cephalosporin C yielded DL-valine and D-α-aminoadipic acid, together with some L-alanine and glycine. On acid hydrolysis alone it yielded D-α-aminoadipic acid but no penicillamine (β-mercaptovaline). It thus appeared that cephalosporin C contained the carbon–nitrogen skeleton of penicillamine, but that it did not contain the characteristic 4-carboxy-5:5-dimethylthiazolidine ring of the true penicillins.

This paper describes two degradation products of cephalosporin C which are derived from the side chain and part of the nucleus to which the latter is attached. One product is apparently δ-amino-δ-carboxyvalerylglycine (I), which has been obtained earlier from cephalosporin N (Newton & Abraham, 1954; Abraham & Newton, 1954). The other is 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (II) and results from a type of fission that has not been observed with cephalosporin N or other penicillins.

When cephalosporin C was heated in 0·1N-hydrochloric acid at 100° for 16 min., carbon dioxide was evolved and a complex mixture of ninhydrin-positive degradation products was formed. Analysis of the mixture by electrophoresis on paper, followed by chromatography in butan-1-ol–acetic acid–water (Woiwod, 1949), showed that at least three acidic substances, three neutral substances and two basic substances were present. One of the acidic substances corresponded in behaviour with α-aminoadipic acid. All the remaining substances were labile on treatment with acid under more vigorous conditions, being destroyed in N-hydrochloric acid at 100° in 30 min.

The neutral products formed on hydrolysis of cephalosporin C in 0·1N-hydrochloric acid for 16 min. were separated from acidic material by passage through a column of Dowex 1 (×10) (acetate form), and then from basic material by passage through a column of Amberlite XE-64 (pyridine form). Oxidation of the resulting neutral fraction with silver oxide yielded a small amount of an acidic substance which was purified by chromatography on Dowex 1 (×10) (acetate form). This substance appeared to be homogeneous, except for contamination with a trace of α-aminoadipic acid, when examined by electrophoresis and chromatography on paper in a number of different systems (see Experimental). In these systems it was indistinguishable from an authentic sample of D-δ-amino-δ-carboxyvalerylglycine synthesized by Abraham & Newton (1954). On acid hydrolysis it yielded approximately equal amounts of two substances which were indistinguishable from glycine and α-aminoadipic acid respectively. It was concluded that the substance from cephalosporin C was δ-amino-δ-carboxyvalerylglycine (I). Earlier work (Abraham & Newton, 1956) had indicated that the residue of α-aminoadipic acid in cephalosporin C was linked to the rest of the molecule through its δ-carboxyl group.



(I)

When a solution of cephalosporin C in aqueous pyridine (pH 5–7) was kept at 37° a new substance with antibacterial activity was formed. On paper electrophoresis at pH 7 this substance behaved as though it had no net charge. It was subsequently shown to be one of a family of compounds (named the cephalosporin C_A family) whose different members are formed by reaction of cephalosporin C with different heterocyclic bases (Hale, Abraham & Newton, 1958; Abraham & Newton, 1958). After the reaction in aqueous pyridine had been allowed to proceed for 72 hr., some cephalosporin C remained unchanged and migrated towards the anode when the mixture of products was subjected to electrophoresis on paper at pH 7 (Abraham & Newton, 1958). A second acidic substance, which showed no detectable activity against *Staphylococcus aureus* (N.C.T.C. 6571) or *Salmonella typhi* (strain 'Mrs S', Felix & Pitt, 1935), was revealed when the paper was sprayed with ninhydrin. This

substance migrated slightly faster than cephalosporin C, from which it was just resolved. It was also formed when the sodium salt of cephalosporin C was kept in water (pH 7) at 37°, although no cephalosporin C_A was produced under these conditions.

The new acidic substance was isolated from the products obtained when cephalosporin C was kept in aqueous pyridine by chromatography of the mixture on a column of Dowex 1 (×10) (acetate form). Cephalosporin C_A passed rapidly through the column. On elution with 0·5N-acetic acid the new substance emerged in a sharp band which preceded, and was well separated from, a band due to cephalosporin C.

The substance isolated in this way was readily soluble in water at pH 7·5, but separated in crystalline form when the solution was adjusted to pH 3·8. It showed $[\alpha]_D^{20} - 17·6°$ in N-hydrochloric acid. Elementary analysis indicated that a possible molecular formula was $C_9H_{12}O_4N_2S$. The results of electrometric titration were consistent with the presence of a basic group (pK_a 9·9) and two acidic groups (pK_a about 2·6 and 4·0 respectively) in a compound with this molecular formula. The ultraviolet absorption of the substance showed a maximum at 237 mμ in water (log ε 3·78) and at 233 mμ in N-hydrochloric acid (log ε 3·88). The substance appeared to be unchanged by treatment with hot 6N-hydrochloric acid or with 0·1N-sodium hydroxide at room temperature.

These properties, when considered in conjunction with the information relating to the structure of cephalosporin C that had been obtained by Abraham & Newton (1956), suggested that the new substance was the thiazole (II). The absorption of the substance in ultraviolet light was similar to that recorded by Brookes, Fuller & Walker (1957) for 2-(1-amino-2-methylpropyl) thiazole-4-carboxylic acid hydrochloride obtained from micrococcin P. The change in $\lambda_{max.}$ from 237 mμ in water to 233 mμ in N-hydrochloric acid could be attributed to protonization of the heterocyclic nitrogen atom. Dr James Walker informed us that no such change in $\lambda_{max.}$ occurred with 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid, but suggested that in this case protonization of the heterocyclic nitrogen atom is hindered by the proximity of a positively charged amino group.

Support for structure (II) was obtained from an examination, by electrophoresis and chromatography on paper, of the products formed when the substance was subjected to hydrogenolysis under two different conditions, followed by hydrolysis with hot 6N-hydrochloric acid. Hydrogenolysis with hydrogen in the presence of an excess of Adams catalyst led to products which appeared to be glycine (III), alanine (IV), α-aminoadipic acid (V), lysine (VII) and norleucine (VIII) respectively. Two other ninhydrin-positive products were also formed. The first showed a low R_F (R_{Gly} 0·56) [the abbreviations in this subscript and in those below are for amino acids; see *Biochem. J.* (1957), **66**, 8] in butan-1-ol–acetic acid–water and no net charge at pH 7, properties which suggested that it might be the diaminodicarboxylic acid (VI). The behaviour of the second, which showed R_{Lys} 1·4 and migrated slightly less rapidly than lysine towards the cathode at pH 7, suggested that it might be ε-*N*-ethyl-lysine (IX). Hydrogenolysis with Raney nickel led to alanine, a neutral substance with R_{Gly} 0·56 and a third substance with R_{Val} 0·92. The third substance showed the same R_F as pipecolic acid (X). Like the latter, it gave a violet spot which faded to brown–grey when sprayed on paper with ninhydrin, and a green spot when sprayed with a solution of isatin in acetone (Grobbelaar, Zacharius & Steward, 1954). All these products could be accounted for if hydrogenolysis involved fission at two or more points of the thiazole ring, as indicated by the broken lines in (II). The type of fission responsible for the formation of pipecolic acid would be analogous to that which yields isoleucinol from the *N*-terminal 2-(1-amino-2-methylbutyl)thiazoline fragment of bacitracin A (Lockhart, Abraham & Newton, 1955).

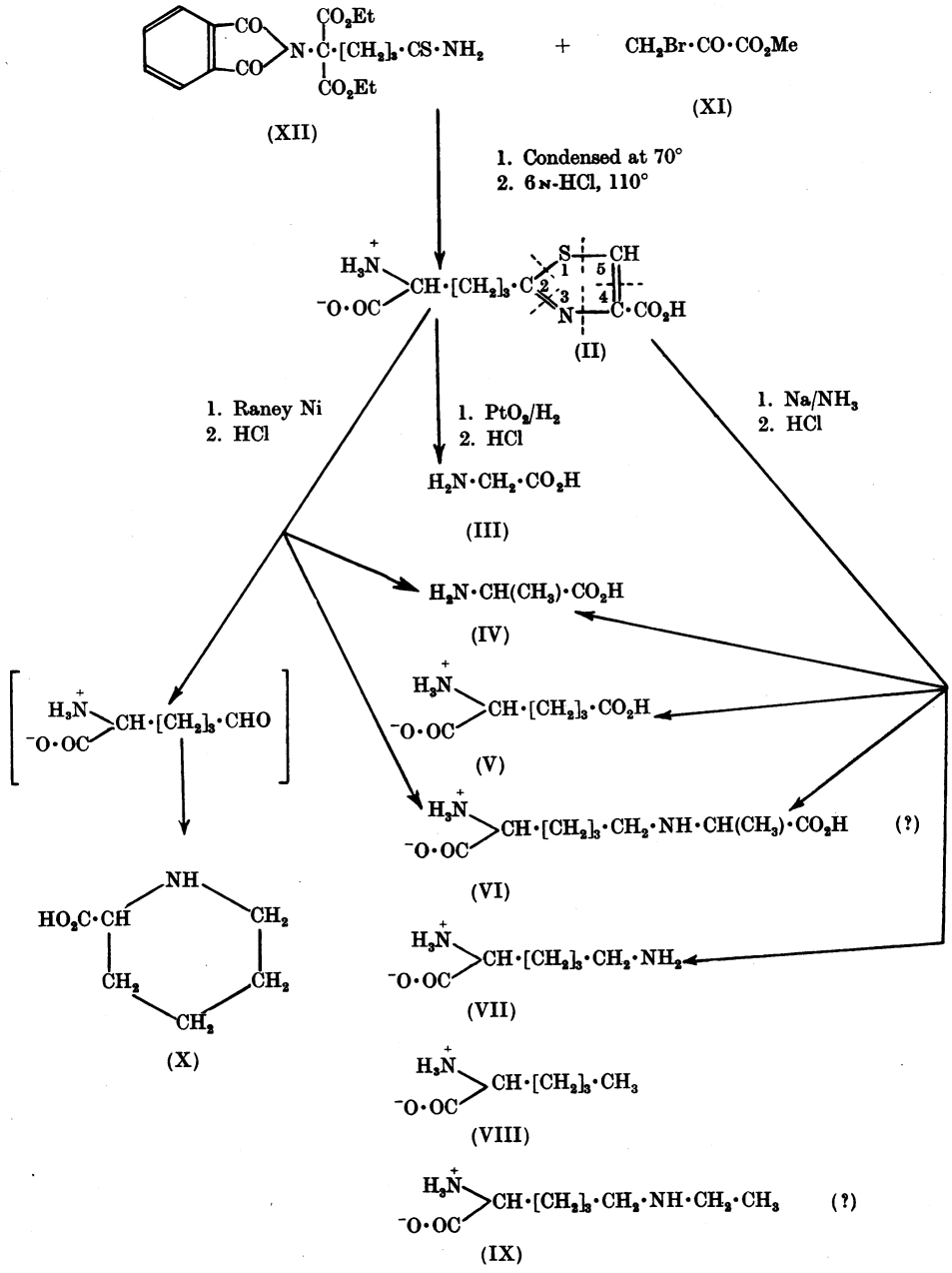
At this stage we were indebted to Dr James Walker for information about a method for the structural study of thiazoles which consisted of reduction with sodium in liquid ammonia followed by acid hydrolysis of the product (Brookes, Clark, Majhofer, Mijović & Walker, 1960). The application of this method to the substance from cephalosporin C yielded products which behaved like alanine, α-aminoadipic acid and lysine respectively, and also a neutral product with R_{Gly} 0·56 in butan-1-ol–acetic acid–water. Fission of (II) to yield these products was consistent with the results of Brookes *et al.* (1960), who obtained alanine and valine from 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid and alanine and *N*-benzylalanine from 2-phenylthiazole-4-carboxylic acid. The fission of 2-phenylthiazole-4-carboxylic acid to yield *N*-benzylalanine would correspond with the fission of the thiazole (II) to yield the compound with R_{Gly} 0·56 if the latter had the structure (VI).

2-(DL-4-Amino-4-carboxybutyl)thiazole-4-carboxylic acid (II) was then synthesized by condensation of methyl bromopyruvate (XI) with the thioamide (XII), or with the corresponding acetamido compound, and hydrolysis of the product.

The thioamide (XII) was prepared from the corresponding phthalimidonitrile (Sörensen, 1903–06) by heating the latter with liquid hydrogen sulphide at 70° in an autoclave (cf. Erne & Erlenmeyer, 1948), and was obtained in crystalline form

after countercurrent distribution in the system benzene–methanol–water (1:1:1, by vol.). Its infrared-absorption spectrum in chloroform showed bands at 1785 and 1722 cm.$^{-1}$ which could be attributed to the phthalimido group (cf. Sheehan, Henery-Logan & Johnson, 1953). Its ultraviolet-absorption spectrum in ethanol showed one maximum at 220 mμ (log ϵ 4·62) attributable to the phthalimido group, and one at 269 mμ (log ϵ 4·02) attributable to the thioamide. Burawoy (1939) reported $\lambda_{max.}$ 265 mμ, log ϵ 4·11 for thioacetamide.

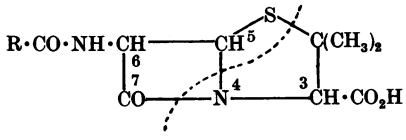
The corresponding acetamidonitrile was prepared from diethyl acetamidomalonate and γ-chlorobutyronitrile and converted into the thioamide in a similar manner. Condensation of either thioamide with methyl bromopyruvate in ethanol at 70° yielded a product from which the optically



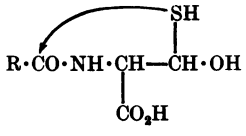
inactive thiazole (II) was formed on hydrolysis with acid.

The synthetic thiazole was indistinguishable from the substance from cephalosporin C when the two products were compared by electrophoresis or chromatography on paper under a variety of conditions. The ultraviolet-absorption spectra of the two compounds (in water or in N-hydrochloric acid) were very similar. The substance from cephalosporin C was racemized by treatment with acetic anhydride under conditions similar to those used by du Vigneaud & Meyer (1932) for the racemization of amino acids. The optically inactive product (m.p. 233–237°) and the synthetic thiazole (m.p. 235–237°) showed no depression in melting point when mixed. X-ray powder photographs of the optically inactive product and the synthetic thiazole were kindly taken by Mrs S. Cole and Dr Dorothy Hodgkin and reported to be identical.

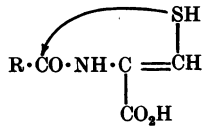
The stable thiazole (II) cannot be present in cephalosporin C itself, since the latter readily yields α-aminoadipic acid on treatment with hot dilute acid. The thiazole ring may therefore be formed by a rearrangement which accompanies a hydrolytic cleavage of cephalosporin C in neutral solution. However, the formation of (I) and (II) suggests that cephalosporin C contains a sequence of atoms which is identical with, or very similar to, that in the *N*-acylcysteine fragment of the true penicillins (XIII). It thus strengthens earlier evidence that cephalosporin C is biogenetically related to cephalosporin N. The sulphur atom and C-5, C-6 and C-7 in the fragment of (XIII) to the left of the dissection shown by the broken line could correspond with a fragment of cephalosporin C which yields the sulphur atom, C-5, C-4 and the carboxyl group respectively of (II). The formation of (I) after acid hydrolysis and oxidation with silver oxide is consistent with the assumption that carbon atoms in cephalosporin C which correspond to C-7 and C-5 in (XIII) form part of a latent labile carboxyl group and a potential aldehyde group respectively. Fission of a sulphur–carbon bond in cephalosporin C and other hydrolytic changes might then lead to transient intermediates such as (XIV) or (XV) from which the thiazole (II) could be formed by intramolecular condensation.



(XIII)



(XIV)



(XV)

EXPERIMENTAL

Elementary analyses were by Weiler and Strauss. Infrared spectra were measured with a Perkin–Elmer Double Beam Photometer Model 21.

Electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14 v/cm. for 2·5 hr.) was carried out as described by Newton & Abraham (1954) in collidine acetate buffer (0·05 M to acetate), pH 7, and in 10% (v/v) acetic acid, pH 2·3. Paper chromatograms were run on Whatman no. 1 paper in the following solvent systems: A, butan-1-ol–acetic acid–water (4:1:4, by vol.) (Woiwod, 1949); B, 80% (w/w) phenol in an atmosphere saturated with 50% (v/v) acetic acid (Dent, 1948); C, butan-1-ol saturated with aq. 0·1 N-HCl; D, butan-2-ol saturated with 3% aqueous NH_3; E, propan-1-ol–water (7:3, v/v); F, triethylamine–acetone–water (1:16:3, by vol.) (Wright & Stadtman, 1956).

In some experiments electrophoresis at pH 7 was followed by chromatography in system A, the solvent being run in a direction perpendicular to that of the current.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1954).

Isolation of δ-amino-δ-carboxyvalerylglycine from cephalosporin C. A solution of cephalosporin C (sodium salt) (100 mg.) in 0·1 N-HCl (5 ml.) was heated at 100° for 16 min. It was then evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950) and the residue kept in a vacuum desiccator overnight in the presence of solid KOH. The dry residue was dissolved in water (2 ml.) and the solution added to a column of Dowex 1 (×10) (5 cm. × 1 cm., 200–400 mesh) in the acetate form. On elution with water, neutral and basic ninhydrin-positive material passed rapidly through the column and emerged in 6 ml. of percolate. The latter was evaporated to dryness *in vacuo* and the residue dissolved in water (2 ml.) and added to a column of Amberlite XE-64 previously washed with an excess of aq. N-pyridine and then with water (50 ml.). On elution with water, neutral ninhydrin-positive material emerged from the column in the first 14 ml. of percolate. The latter yielded 30 mg. of residue on evaporation to dryness *in vacuo*.

The neutral residue was dissolved in water (5 ml.) and the solution stirred with Ag_2O (freshly prepared from 340 mg. of $AgNO_3$) at 80° for 1·5 hr. Ag^+ ions were removed from the supernatant by addition of N-HCl (to pH 4) and the clear solution obtained after centrifuging was evaporated to dryness *in vacuo*. The residue (15 mg.) was dissolved in water (1 ml.) and added to a column of Dowex 1 (×10) (5 cm. × 1 cm., 200–400 mesh) in the acetate form. Neutral material was eluted with water (2 ml.). Elution was then continued with 0·5 N-acetic acid, 1 ml. fractions being collected. An acidic ninhydrin-positive substance (3 mg.) emerged in fractions 5 and 6.

This substance was indistinguishable from synthetic D-δ-amino-δ-carboxyvalerylglycine (Abraham & Newton, 1954)

when subjected to electrophoresis or chromatography on paper under various conditions. On electrophoresis in collidine acetate (pH 7) both products migrated somewhat less far (6·8 cm.) towards the anode than α-aminoadipic acid (8·0 cm.) and on electrophoresis in 10% (v/v) acetic acid they migrated slightly less far (4·2 cm.) towards the cathode than α-aminoadipic acid (5·3 cm.). On chromatography in butan-1-ol–acetic acid–water and in phenol–acetic acid both products showed R_F values of 0·16 and 0·31 respectively. Hydrolysis of the product from cephalosporin C with N-HCl at 110° for 16 hr. yielded material which showed two ninhydrin-positive spots of similar intensity after electrophoresis and chromatography on paper. One of these spots was indistinguishable from a spot due to glycine, and the other from a spot due to α-aminoadipic acid, after electrophoresis at pH 7 and chromatography in systems A and F.

Isolation of 2-(D-4-*amino*-4-*carboxybutyl*)*thiazole*-4-*carboxylic acid from cephalosporin C.* To a stirred solution of cephalosporin C (sodium salt) (1·0 g.) in water (50 ml.) was added Dowex 50 (×8) (200–400 mesh, H form) until the pH fell to 2·5. About 680 mg. of damp-dry resin was required. The resin was removed by filtration and pyridine (4 ml.) was added to the resulting solution of cephalosporin C (free acid). The mixture was kept at 37° for 72 hr. and then freeze-dried. The residue was stirred with acetone (about 50 ml.) to remove traces of pyridine and the remaining solid was separated by centrifuging and freed from acetone *in vacuo*. The resulting powder (750 mg.) was dissolved in water (2 ml.) and added to a column of Dowex 1 (×10) (25 cm. × 2 cm., 200–400 mesh) in the acetate form. Neutral material was eluted with water, 5 ml. fractions being collected. The neutral cepholosporin C_A (Abraham & Newton, 1958; Hale *et al.* 1958) emerged in fractions 4–6. Elution was then continued with 0·5N-acetic acid, 25 ml. fractions being collected. A sharp band of ninhydrin-positive material emerged in fractions 25–30. A broader band due to unchanged cephalosporin C emerged in fractions 60–80. After neutralization with NaOH this yielded 165 mg. of cephalosporin C (sodium salt).

Fractions 25–30 were combined and the solution was evaporated to dryness *in vacuo*. The remaining white microcrystalline solid (46 mg.) was mixed with water (0·2 ml.) and aq. N-NH_3 added until the solid had completely dissolved (pH 8). 4N-Acetic acid was then added to the clear solution in an amount sufficient to lower its pH to about 3·8. Within a few seconds the product began to separate in crystalline form. After 2 hr. the mixture was filtered and the product washed with a little water and dried in a vacuum desiccator (38·6 mg.). The off-white product lost no further weight on drying at 56° in a high vacuum. It had m.p. 245–246° (decomp.), $[\alpha]_D^{20}$ −17·6° in N-HCl (*c*, 1·3) (Found: C, 43·8; H, 5·0; N, 10·9; S, 12·3%; equiv. 250. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·5; S, 13·1%; equiv. 244). The presence of a small amount of pigmented impurity, which was not removed by recrystallization, may have been responsible for the low values found for N and S. The pigment was lost during procedures, described below, that were used to obtain the compound in a racemic form, and satisfactory analytical data were obtained with the racemate. The ultraviolet-absorption spectrum of the optically active product showed $\lambda_{max.}$ 237 mμ (log ε 3·78) in water and $\lambda_{max.}$ 233 mμ (log ε 3·88) in N-HCl.

The product was poorly soluble in water between pH 3 and 5, but was readily soluble in N-HCl or at pH values higher than 7. Electrometric titration in water at 20° indicated that it contained ionizable groups with pK_a values of about 2·6, about 4·0, and 9·9 respectively. When subjected to electrophoresis on paper at pH 7 it migrated less far (5·5 cm.) towards the anode than α-aminoadipic acid (8·0 cm.) but further than cephalosporin C (4·8 cm.) (spots revealed by ninhydrin). On paper chromatograms run in system A it showed an R_F slightly greater than that of α-aminoadipic acid, the two compounds just being resolved when run together. These properties were unchanged by keeping the product 16 hr. in 0·1N-NaOH at 20° or in N-HCl at 105°.

Degradations of the thiazole from cephalosporin C. (*a*) *Hydrogenolysis in the presence of platinum dioxide.* A solution of the thiazole (5 mg.) in 0·5N-acetic acid (15 ml.) was shaken with Adams catalyst (25 mg., L. Light and Co. Ltd.) under H_2 at room temp. for 22 hr. The mixture was filtered and the filtrate evaporated to dryness. The residue was heated in 6N-HCl (0·5 ml.) for 16 hr. at 105° and the solution evaporated to dryness. The resulting material was then examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. Spots were coloured with ninhydrin, and in the following account the relative intensity of the spots is indicated by the number of + signs in parentheses. Of the products which showed no net charge at pH 7 three had R_F values corresponding to glycine (+), alanine (+ + +) and norleucine (+) respectively and a fourth substance (+ +) which was possibly (VI), showed R_{Gly} 0·56. Two basic products were observed, one of which corresponded to lysine (+ + +) in mobility and R_F and another (+) which migrated somewhat less far (10·0 cm.) than lysine (12·0 cm.) towards the cathode and showed R_{Lys} 1·4. The latter was conceivably (IX). One acidic product was observed (+ + +) which corresponded in mobility and R_F with α-aminoadipic acid.

(*b*) *Hydrogenolysis with Raney nickel.* The thiazole (6 mg.) was mixed with water (1 ml.) in a tube fitted with a reflux condenser and 0·1N-NaOH added until a clear solution was obtained (pH 7·5). About 50 mg. of Raney nickel (Pavlic & Adkins, 1946) was added and the tube immersed in an oil bath at 160° for 20 min. The insoluble material was spun down and washed twice with water. The combined supernatant and washings were shaken twice with 1 vol. of 1% (w/v) oxine in $CHCl_3$ and then twice with 1 vol. of $CHCl_3$ alone. The aqueous solution was evaporated to dryness *in vacuo* and the residue (5 mg.) heated in 6N-HCl (0·5 ml.) at 110° for 16 hr. The HCl was removed *in vacuo* and the residue examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. Three spots were revealed by ninhydrin, all of which were due to substances that showed no net charge at pH 7. The R_F of one spot (+ + +) corresponded with that of alanine. A second spot (+ +) showed R_{Gly} 0·56 (possibly due to V 1) and a third spot (+ +) showed R_{Val} 0·92. The third spot was violet at first but faded to brown–grey within 36 hr. It was not resolved from spots due to authentic DL-pipecolic acid in system A or B and in the latter showed R_{Val} 1·15. It was coloured green when the papers were sprayed with 0·2% isatin in acetone and heated for 5 min. at 100°. Pipecolic acid yielded a green spot under similar conditions.

(*c*) *Treatment with sodium in liquid ammonia.* The thiazole (20 mg.) was added to liquid NH_3 (5 ml.). Small pieces of

Na were added until a persistent blue colour appeared and the colour was then discharged by addition of a crystal of NH_4Cl. The solution was allowed to evaporate to dryness and the residue was heated in 6N-HCl (10 ml.) for 16 hr. at 110°. The HCl was removed *in vacuo* and the residue examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. The material with no net charge at pH 7 showed two spots, one (++) with the same R_F as that of alanine and the other (++) with R_{Gly} 0·56 (possibly due to VI). Two spots due to material which migrated towards the anode corresponded in position with α-aminoadipic acid (++) and unchanged thiazole respectively. A spot (++) due to material which migrated towards the cathode corresponded in position with lysine.

Racemization of the thiazole from cephalosporin C. The thiazole (50 mg.) was dissolved in 0·91N-NaOH (0·4 ml.) Acetic anhydride (0·28 ml.) was added drop by drop with vigorous shaking. The mixture was kept at 37° in a sealed tube for 20 hr. and then evaporated to dryness in a rotary evaporator. The remaining gum was dissolved in water (0·6 ml.) and 0·4 ml. of N-HCl was added. No precipitate formed and the solution was again evaporated to dryness, yielding a gum and crystals of NaCl. The gum was dissolved in acetone–ethanol (about 1:1, v/v) and the clear solution re-evaporated. The amorphous residue was heated in 3N-HCl (2 ml.) for 16 hr. at 105° and the solution evaporated *in vacuo*. The residue was dissolved in water (1 ml.) and added to a column of Dowex 1 (×10) (1 cm. × 25 cm., 200–400 mesh) in the acetate form. Elution was carried out with 0·5N-acetic acid, 5 ml. fractions being collected. A major band of material (as gauged by absorption at 237 mμ) emerged in fractions 21–31. These fractions were evaporated in a rotary evaporator and yielded a crystalline residue. The latter was mixed with water (0·2 ml.) and about 0·4 ml. of aq. 0·4N-NH_3 added, when the crystals dissolved to give a clear solution. 4N-Acetic acid was added until the pH fell to about 3·8. After a short interval the product separated in crystalline form, and was collected, washed with a little water, and dried *in vacuo* at 100° (21 mg.). It showed no detectable optical activity in N-HCl (*c*, 3·7; l, 0·5 dm.). It melted at 233–237° and showed $\lambda_{max.}$ at 237 mμ in water (log ε 3·80). It could not be distinguished from the original optically-active thiazole by electrophoresis on paper at pH 7 or chromatography on paper in system A (Found: C, 43·9; H, 5·1; N, 11·7; S, 13·4. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·5; S, 13·1%.)

Synthesis of 5-acetamido-5:5-diethoxycarbonylvaleronitrile. Diethyl acetamidomalonate (1·37 g.) was dissolved in 2 ml. of ethanol (dried with Mg and freshly distilled) in a Pyrex test-tube (12 cm. × 3 cm. diam.) with a B 24 ground-glass joint. To this solution was added a solution of Na (0·145 g., 1 mol.prop.) in ethanol (1·5 ml.). The resulting solution (protected by a soda-lime tube) was warmed in an oil bath at 60–70° for 15 min., during which time a white precipitate appeared. The mixture was then evaporated *in vacuo* to a syrup, the Pyrex tube being used as the distillation flask of a rotary evaporator. The evaporator was run for a further 2 hr. while the syrup was heated to about 60° by means of an infrared lamp. This led the syrup to crystallize. The solid was scraped from the walls of the tube and γ-chlorobutyronitrile (1·23 g., 1·9 mol.prop.) (L. Light and Co., Poyle, Bucks) was added. An air-cooled reflux condenser was then fitted, and the tube heated in an oil bath at 165–175° so that the γ-chlorobutyronitrile refluxed. After 17 hr., the reaction mixture, which was no longer alkaline, was allowed to cool to room temperature and stirred with dry ethanol (20 ml.). The solution was filtered, and the solid washed twice with 10 ml. portions of boiling ethanol. The filtrates were combined and evaporated *in vacuo* in a rotary evaporator, the flask being finally heated by means of an infrared lamp (to about 60°) so that much of the excess of γ-chlorobutyronitrile was removed. The residue was purified by countercurrent distribution in the system *n*-butyl acetate–water, 15 ml. of upper phase and 20 ml. of lower phase being used and the upper phase being mobile. After 100 fundamental transfers, 45 withdrawals of upper phase were made from tube 100. Dry weights were determined on samples (0·5 ml.), first of every fifth withdrawn fraction, and subsequently of every second fraction in regions close to peaks of material. Some material (possibly unchanged γ-chlorobutyronitrile) was present in the early withdrawn fractions, the weight of this material declining from a maximum in the first withdrawal to zero in the ninth. The main band of material was found in withdrawn fractions 16–35 and reached a maximum in fraction 26. Samples of material from these fractions were hydrolysed by heating in 6N-HCl for 12 hr. at 110°, and the hydrolysates examined by paper electrophoresis in collidine acetate (pH 7) and by paper chromatography in system A, the spots being developed with ninhydrin. All the hydrolysates contained α-aminoadipic acid together with a trace of neutral material, but those from fractions 16–22 contained in addition a small amount of an unidentified amino acid which moved a little faster than α-aminoadipic acid both on electrophoresis and chromatography. The occurrence of this material correlated closely with a small hump in the leading edge of the main band of the dry-weight curve. Fractions 23–32 were therefore combined and evaporated to dryness *in vacuo* in a rotary evaporator. The syrup so obtained crystallized during the course of the next week, and was recrystallized from *cyclo*hexane as colourless needles, m.p. 51–52° (0·5 g., 28%) (Found: C, 54·8; H, 7·5; N, 9·8. $C_{13}H_{20}O_5N_2$ requires C, 54·9; H, 7·1; N, 9·9%).

Synthesis of 5:5-diethoxycarbonyl-5-phthalimidovalerothioamide (thioamide A). Diethyl phthalimidomalonate was prepared according to the procedure recommended by Osterberg (1944) and converted by the method of Sörensen (1903–6) into 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile, which was recrystallized from di*iso*propyl ether.

Hydrogen sulphide, generated in a Kipps apparatus from FeS and 4N-HCl, was freed from acid spray by bubbling through water, dried by passage through a $CaCl_2$ tower, and condensed by passing into a Pyrex tube cooled to −70° in a mixture of ethanol and solid CO_2. Liquid H_2S required for the reaction was then transferred as required to the reaction vessel, connected to this reservoir, by removing the cold bath and replacing it around the reaction vessel.

Into 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile (1·92 g.) and triethylamine (about 0·2 ml.) was condensed liquid H_2S (15 ml.). The Pyrex tube containing this mixture was quickly transferred to a stainless-steel bomb, which was sealed and immersed in water at 75° in a lagged polythene bucket. The rate of cooling was such that the water temperature fell to 45° in 5 hr. The bomb was kept in the bucket for a total of 50 hr. The pressure was then released, excess of H_2S allowed to evaporate, and residual volatile

material was removed from the syrupy product by warming to 70° under water-pump vacuum. The residue was dissolved in benzene (about 40 ml.), washed with 0·2M-KH_2PO_4 (about 20 ml.) and then with water, and the benzene solution evaporated to dryness in a rotary evaporator. The syrup so obtained was purified by countercurrent distribution in the system methanol–benzene–water (1:1:1, by vol.), 20 ml. of lower layer and 19 ml. of upper layer being used in each tube and the upper layer being mobile. After 100 fundamental transfers, 70 withdrawals were made from the 101st tube. Samples (2 ml.) were taken from every fifth withdrawn fraction and dry weights determined. Further samples were then taken in the neighbourhood of dry-weight peaks. Dry weight was negligible in the withdrawn fractions beyond the fiftieth, the main peak lying in the twelfth withdrawn fraction ($K = 8$), and a very small peak occurring in the region of the thirty-seventh withdrawal ($K = 2·7$). Withdrawn fractions 8–17 were combined and evaporated to dryness in a rotary evaporator, giving a syrup (approximately 500 mg., yield 25%) which crystallized during the course of the next few days. Recrystallization from CCl_4 and a little $CHCl_3$ gave pale ivory-coloured crystals, m.p. 143–145°, raised on recrystallization from ethanol–water to 144–145°. The infrared spectrum of the compound ($CHCl_3$ solution) showed peaks at 1785 and 1722 cm.$^{-1}$, consistent with the presence of the phthalimide ring (Sheehan *et al.* 1953). The ultraviolet-absorption spectrum (ethanol solution) showed a maximum at 220 mμ (log ε 4·62), in which it resembled that of the parent nitrile ($\lambda_{max.}$ 220 mμ, log ε 4·6) but differed from that of phthalic acid and dimethylphthalate, which show a weaker maximum at 225 mμ (log ε = 3·87). A second maximum at 269 mμ (log ε 4·02) was consistent with the presence of the thioamide group (Burawoy, 1939). The compound thus appeared to be 5:5-*diethoxycarbonyl*-5-*phthalimidovalerothioamide* (Found: C, 56·1; H, 5·7; N, 7·2; S, 8·0. $C_{19}H_{22}O_6N_2S$ requires C, 56·1; H, 5·5; N, 6·9; S, 7·9%).

Synthesis of thioamide B. In a previous experiment, 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile (1·5 g.) was similarly treated with liquid H_2S in a bomb for 14 hr. The product was freed from volatile material as described above, but the syrup was then subjected to countercurrent distribution in the system benzene–methanol–water (1:1:1, by vol.) without first being dissolved in benzene and washed with buffer solution. In this case, only 24 fundamental transfers were carried out, and 20 withdrawals made from tube 24. Analysis by dry weight revealed a band in the first four withdrawn fractions. The first fraction was found to contain largely unchanged nitrile. The following three fractions were not worked up, but presumably contained thioamide A. Overlapping the first withdrawn band was a broad band with peak in the region of the seventh withdrawal ($K = 3$). The seventh to twelfth withdrawn fractions were combined and evaporated in a rotary evaporator. The syrup so obtained crystallized on standing, and was easily recrystallized from a little ethanol at −10°, giving needles, m.p. 124–126° (500 mg., 30%). After a second recrystallization from a mixture of $CHCl_3$, CCl_4, and a little hexane the product (thioamide B) melted at 123·5–124·5°. Its infrared spectrum ($CHCl_3$ solution) showed bands at 1671 and 1578 cm.$^{-1}$, consistent with the presence of a secondary amide group and a band at 1735 cm.$^{-1}$ which could be attributed to ester groups. The absence of bands at 1785 and 1722 cm.$^{-1}$ indicated that the phthalimide ring was not present. The ultraviolet spectrum of the product (ethanol solution) showed a plateau at 220–225 mμ resembling the broad maximum at 225 mμ (log ε 3·86) shown by phthalic acid and its dimethyl ester. The presence of the thioamide group was indicated by a maximum at 270 mμ (log ε 4·02) (cf. Burawoy, 1939). This evidence seemed consistent with the structure 5-(*o*-carboxybenzamido)-5:5-diethoxycarbonylvalerothioamide for thioamide B (Found: C, 54·2; H, 6·1; N, 6·6; S, 7·7. $C_{19}H_{24}O_7N_2S$ requires C, 53·7; H, 5·7; N, 6·6; S, 7·6%). However, the compound contained no group which could be titrated in 80% (v/v) ethanol between pH 2 and pH 11, and attempts to precipitate salts from an ethereal solution of the compound by addition of anhydrous bases were unsuccessful. The structure of thioamide B thus remains uncertain.

Synthesis of 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. 1. *From 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile without isolation of the thioamide.* The nitrile (300 mg.) was treated with liquid H_2S and a catalytic amount of triethylamine, as described above. Volatile material was removed by warming under water-pump vacuum, and to the syrupy residue was added a solution of methyl bromopyruvate (approx. 200 mg.) (Kuhn & Dury, 1951) in dry ethanol (2 ml.). The mixture was warmed to 70° for 1 hr., and allowed to stand at room temperature overnight. The ethanol was evaporated in a stream of air, the residue dissolved in acetic acid (0·5 ml.) and the solution transferred to a small Pyrex tube. After addition of an equal volume of 11N-HCl, the tube was sealed and heated at 110° for 16 hr. The solvent was evaporated by heating at 100° in a stream of air. The residue was dissolved in 0·5N-acetic acid (2 ml.) and applied to a column (26 cm. × 1 cm. diam.) of Dowex 1 (×10) (acetate form, 200–400 mesh). Elution was carried out with 0·5N-acetic acid, thirty-nine 2 ml. fractions and then thirty-two 5 ml. fractions being collected. Every second fraction was examined by ninhydrin reaction and by absorption at 237 mμ. A sharp band of ninhydrin-positive material (which behaved as α-aminoadipic acid when examined by electrophoresis and chromatography on paper) emerged in fractions 4–15. Fractions 41–54 contained the only other ninhydrin-positive band, and showed absorption at 237 mμ. Fractions 43–52 were combined and evaporated to dryness in a rotary evaporator. The solid so obtained was dissolved in water (0·5 ml.) by the dropwise addition of aq. N-NH_3 solution (to pH 7·5) and a few undissolved particles were removed by centrifuging. The thiazole was then recrystallized by adjusting the solution to pH 3·8 with 2N-acetic acid. Yield, 93 mg. (47%). The thiazole had m.p. 235–237° (decomp.); $\lambda_{max.}$ (water) 237 mμ (log ε 3·79); $\lambda_{max.}$ (N-HCl) 233 mμ, (log ε 3·91). The change in $\lambda_{max.}$ and $\epsilon_{max.}$ occurs gradually over the range 0·5–0·2N-HCl (Found: C, 43·9; H, 4·9; N, 11·1; S, 12·6. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·4; S, 13·1%).

2. *From 5-acetamido-5:5-diethoxycarbonylvaleronitrile without isolation of the thioamide.* To the nitrile (32 mg.) was added a drop of triethylamine, and approximately 5 ml. of liquid H_2S, and the mixture was heated in a bomb for 14 hr., according to the procedure described above. The product was freed from volatile material, and condensed with methyl bromopyruvate (approximately 100 mg.), and the condensation products were hydrolysed, as in the previous experiment. Electrophoresis on paper at pH 7·0 and chromatography on paper in system A indicated that

the hydrolysate contained the required thiazole (indistinguishable from the product obtained in the previous experiment, and from that obtained from cephalosporin C) together with an almost equal amount of α-aminoadipic acid.

3. *From thioamide A.* Thioamide A (9·3 mg.) was condensed with methyl bromopyruvate (56 mg.) in ethanol solution (0·5 ml.), and the products were hydrolysed as described above. The hydrolysate was evaporated to dryness and the residue examined by chromatography on paper in system A and electrophoresis on paper at pH 7·0. This indicated that the residue consisted of the required thiazole and a trace of α-aminoadipic acid. Comparison of the intensity of the spot (coloured with ninhydrin) corresponding to the thiazole with the intensities of spots obtained with known amounts of the thiazole from cephalosporin C indicated that the synthetic thiazole had been formed in almost quantitative yield.

4. *From the thioamide B.* Thioamide B (9·3 mg.) and methyl bromopyruvate (7 mg.) were condensed in ethanol solution (0·5 ml.) and the products hydrolysed in the manner described above. After evaporation of the hydrolysate to dryness, the residue was examined by electrophoresis on paper at pH 7·0 and chromatography on paper in system A. This indicated that the residue consisted of the required thiazole and a trace of α-aminoadipic acid. Comparison of the intensity of the ninhydrin colour with that given by known concentrations of the thiazole isolated from cephalosporin C indicated that the synthetic thiazole had been formed in almost quantitative yield.

Behaviour on paper chromatography of 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. The synthetic thiazole and the thiazole from cephalosporin C were indistinguishable on paper chromatography in systems A, B, C, D and E. The following R_{Gly} values were obtained in systems A–D: A, 1·76; B, 1·37; C, 3·5; D, 0·56. In system E R_{Gly} was 2·12 when the specimen was applied to the paper in N-HCl and 0·73 when it was applied in aq. 3% (w/v) NH_3, glycine being applied in neutral solution in each case.

SUMMARY

1. A mixture of acidic, neutral and basic products was formed when cephalosporin C was subjected to mild hydrolysis in hot dilute acid. Oxidation of the neutral material with silver oxide yielded an acidic product which behaved like authentic δ-amino-δ-carboxyvalerylglycine on paper chromatography or electrophoresis, and which gave α-aminoadipic acid and glycine on hydrolysis with N-hydrochloric acid at 110°. δ-Amino-δ-carboxyvalerylglycine had previously been obtained from cephalosporin N [(D-4-amino-4-carboxybutyl)penicillin] under similar conditions.

2. An acidic compound, formed when cephalosporin C was kept in neutral aqueous solution at 37°, was isolated by chromatography on an anion-exchange resin. The physical and chemical properties of this compound, including its behaviour on hydrogenolysis, suggested that it was 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. The compound was racemized by treatment with acetic anhydride in aqueous solution.

3. 2-(DL-4-Amino-4-carboxybutyl)thiazole-4-carboxylic acid was synthesized from methyl bromopyruvate and either 5:5-diethoxycarbonyl-5-phthalimidovalerothioamide or the corresponding acetamido compound, the thioamides being prepared from the corresponding nitriles. The synthetic compound was shown to be identical with the racemized product from cephalosporin C.

4. Some implications of these findings are discussed.

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