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Structure and Synthesis of Cephalosporidine, a Degradation Product of Cephalosporin C

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Radioactive cephalosporin C which had been formed by a species of *Cephalosporium* in a medium containing [1^{14}C]acetate yielded a previously unrecognized degradation product on hydrogenolysis with Raney nickel in hot neutral aqueous solution (Trown, Abraham, Newton, Hale & Miller, 1962). The new degradation product was ninhydrin-negative but radioactive and was first detected as a spot on radioautographs after electrophoresis and chromatography on paper of the mixture of substances formed when hydrogenolysis was followed by acid hydrolysis. Subsequently, it was revealed on paper by the chlorination procedure of Rydon & Smith (1952). It showed no net charge at pH 4.5 and its R_F in butan-1-ol-acetic acid-water (4:1:4, by vol.) was 0.78 that of valine. The isolation and nature of this compound are described in the present paper. It has been given the trivial name 'cephalosporidine'.

Although cephalosporidine was first obtained from a mixture of substances formed on hydrogenolysis of cephalosporin C, it was later shown to be formed when cephalosporin C (either sodium salt or free acid) was heated under reflux in aqueous solution. It also appeared to be formed under similar conditions from an impure preparation of the sodium salt of penicillin N (cephalosporin N), and to be identical with a ninhydrin-negative compound which was detected by Trown *et al.* (1962) among the products of hydrolysis of material which probably contained the penillic acid from penicillin N. This material had been separated from cephalosporin C during the purification of the latter on a column of Amberlite XE-58.

Cephalosporidine survived unchanged when the solution in which it had been formed from cephalosporin C was made 2N with respect to hydrochloric acid and then heated under reflux for 16 hr. It was isolated from the acid hydrolysate by adsorption on Dowex 50 (X8) and elution at pH 7, counter-current distribution in a solvent system composed of phenol and 5% (v/v) acetic acid, and chromatography on Dowex 50 (X8) in N-hydrochloric acid followed by 2N-hydrochloric acid. The purified product separated from ethanol-acetone in orthorhombic crystals.

Cephalosporidine formed a monohydrate in moist air. Elementary analysis and an estimate of molecular weight indicated that the probable molecular formula of the monohydrate was $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_2 \cdot \text{H}_2\text{O}$. No C-methyl group was revealed by a Kuhn-Roth determination. The compound showed $[\alpha]_D^{20} - 2^\circ$ (c 10 in water) and $[\alpha]_D^{20} + 7.3^\circ$ (c 10 in 6N-hydrochloric acid). Its ultraviolet-absorption spectrum showed λ_{max} 211 m μ when measured in water and λ_{max} 212 m μ when measured in N-hydrochloric acid (log ϵ , 3.75). When measured in 0.1N-sodium hydroxide it showed λ_{max} 218 m μ (log ϵ , 3.60).

Electrometric titration indicated that cephalosporidine contained two ionizable groups with pK values of less than 2.2 and of 8.1 respectively. These values were consistent with the behaviour of the compound when it was subjected to electrophoresis on paper. It showed no significant net charge at pH 4.5 or 7.0, migrated 0.65 times as far as valine and 0.7 times as far as 1-imidazolylacetic acid towards the cathode at pH 2.2, and migrated

0.5 times as far as 1-imidazolylacetic acid towards the anode at pH 8.9. The presence of a strong band at 1605 cm^{-1} in its infrared-absorption spectrum was consistent with the presence of an ionized carboxyl group.

Cephalosporidine was resistant to hot 0.1N-sodium hydroxide as well as to hot acid. It failed to absorb hydrogen in the presence of Adams platinum oxide catalyst in aqueous or acetic acid solution. An attempt to convert it into an *N*-acetyl derivative by treatment with acetic anhydride was unsuccessful.

These properties, considered in conjunction with those of cephalosporin C (Abraham & Newton, 1961), suggested that cephalosporidine should be assigned the structure (I). This structure, consisting of a fused imidazole-piperidine-2-carboxylic acid ring system, is a 5-carboxy derivative of 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridine. A number of compounds containing the imidazo[1,2-*a*]pyridine (pyrimidazole) ring system are known (Bower, 1957). Hydrogenation of 2-methylimidazo[1,2-*a*]pyridine was stated to yield the 5,6,7,8-tetrahydro derivative (Kondo & Mitsugi, 1937), but no data on the ultraviolet-absorption spectrum of this product were reported.

Structure (I) was consistent with the stability of cephalosporidine to acid and alkali and with its ultraviolet-absorption spectrum in water, *N*-hydrochloric acid and 0.1N-sodium hydroxide. The ultraviolet-absorption spectra of imidazole showed λ_{max} 207 μ m in water ($\log \epsilon$, 3.70), λ_{max} 208 μ m in *N*-hydrochloric acid ($\log \epsilon$, 3.70) and λ_{max} 217 μ m in 0.1N-sodium hydroxide ($\log \epsilon$, 3.36) (cf. Leandri, Mangini, Montanari & Passerini, 1955). 1-Imidazolylacetic acid showed λ_{max} 209 μ m in water, λ_{max} 210 μ m in *N*-hydrochloric acid ($\log \epsilon$, 3.78) and λ_{max} 217 μ m in 0.1N-sodium hydroxide ($\log \epsilon$, 3.65). Subsequently it was shown that the proton-magnetic-resonance spectrum of cephalosporidine could be interpreted satisfactorily in terms of structure (I) (Bishop & Richards, 1963).

The expected pK_a values for the ionizable groups in structure (I) were similar to the values obtained for cephalosporidine. 1-Imidazolylacetic acid was found by electrometric titration to have pK_a

values of less than 2.2 and of 7.1 respectively. Results given by Dedichen (1906) indicate that the electron-repelling effect of an alkyl substituent in position 2 of an imidazole ring [C-9 in (I)] increases the basicity of the ring by about 1 pK_a unit. Thus the pK_a for the imidazole ring in structure (I) should be about 8.1.

The 5-substituted 1-methylimidazole ring in pilocarpine was degraded by oxidation with hydrogen peroxide or permanganate to yield ammonia and methylamine (Pinner & Kohlhammer, 1900). The imidazole ring in pent-2-enyl-penillamine underwent fission when the substance was treated with bromine water (Cook, 1949). Attempts were therefore made to degrade cephalosporidine with these reagents.

After treatment with 30% (w/v) hydrogen peroxide for 24 hr. at room temperature, much of the original cephalosporidine appeared to remain unchanged. Treatment of the resulting material with hot *N*-hydrochloric acid, however, yielded a very small amount of a ninhydrin-positive substance which behaved like α -aminoadipic acid when subjected to chromatography and electrophoresis on paper. Treatment of cephalosporidine with potassium permanganate in dilute aqueous solution at room temperature gave material which was ninhydrin-negative on paper. When this material was hydrolysed in *N*-hydrochloric acid at 105° for 18 hr. two ninhydrin-positive compounds were formed which behaved like α -aminoadipic acid and glycine respectively on paper chromatography and electrophoresis. The formation of the major product, α -aminoadipic acid, was to be expected under these conditions from the piperidine ring of structure (I). Glycine could have been formed from a nitrogen atom and two carbon atoms of the imidazole ring [C-2 and C-3 in (I)].

Cephalosporidine yielded ninhydrin-positive material on oxidation with bromine water. When this material was subjected to paper chromatography and electrophoresis it was found to contain one substance which behaved like α -aminoadipic acid and another which behaved like a synthetic specimen of δ -(α -aminoadipoyl)glycine (Abraham & Newton, 1954). The products formed on hydrolysis of the mixture with *N*-hydrochloric acid at 105° for 24 hr. appeared to include α -aminoadipic acid and glycine.

These reactions showed that cephalosporidine contains the carbon-nitrogen skeleton of α -aminoadipic acid linked through a second nitrogen to a C_2 fragment, as required by (I), and indicated that it is formed from the partial structure (II) which is common to cephalosporin C and penicillin N. Radioactive cephalosporidine would be expected to be formed from cephalosporin C that had been produced in the presence of [$1\text{-}^{14}\text{C}$]acetate, since

this cephalosporin C contains ^{14}C in C-1 and C-6 of its α -aminoadipic acid residue (Trown *et al.* 1962).

(II)

Hydrolysis of cephalosporin C for a short period with hot dilute acid results in the production of carbon dioxide and ammonia, from the carbonyl group and nitrogen atom respectively of the β -lactam ring, and the formation of a neutral compound which is presumably δ -(α -aminoadipoyl)-aminoacetaldehyde since it yields δ -(α -aminoadipoyl)glycine on oxidation with silver oxide (Jeffery, Abraham & Newton, 1960). Similar reactions probably occur when cephalosporin C is heated in neutral aqueous solution. Hydrolysis of penicillin N leads to a thiazolidine in which δ -(α -aminoadipoyl)aminoacetaldehyde is condensed with β -thiolvaline (Newton & Abraham, 1954). It seemed possible that δ -(α -aminoadipoyl)aminoacetaldehyde, in the free state or liberated from a thiazolidine, would yield (I) by intramolecular condensation in the manner shown in (III). The process would be analogous to one which has been used for the synthesis of imidazoles (Cornforth, 1949). An alternative mechanism was considered, according to which the nitrogen atom in the β -lactam ring of cephalosporin C was incorporated into an imidazole ring and then displaced $-\text{NH}_3^+$ from the amino group of (II). This mechanism, however, could not readily be applied to the formation of cephalosporidine from penicillin N, for the nitrogen atom in the β -lactam ring of the latter would be expected to be retained in the β -thiolvaline fragment of the molecule.

(III)

The finding that cephalosporidine was optically active thus suggested that it had retained at C-5, at least in part, the D-configuration of the residue of α -aminoadipic acid in cephalosporin C. However, the change from a negative rotation in water

to a positive rotation in 6N-hydrochloric acid was the reverse of that shown by D- α -amino acids which conform to the Clough-Lutz-Jirgensons rule.

To test the hypothesis that δ -(α -aminoadipoyl)-aminoacetaldehyde was an intermediate in the formation of cephalosporidine from cephalosporin C, DL- δ -(α -aminoadipoyl)aminoacetaldehyde diethylacetal was synthesized. In a preliminary small-scale experiment the procedure used by Abraham & Newton (1954) for the synthesis of D- δ -(α -aminoadipoyl)glycine was followed. δ -Ethyl DL-benzoyloxycarbonyl- α -aminoadipate was converted, through the hydrazide, into the corresponding azide, and the latter coupled with aminoacetal. Removal of the benzoyloxycarbonyl group by catalytic hydrogenation yielded a product which appeared to be the required compound, since mild acid hydrolysis followed by oxidation with silver oxide converted it into δ -(α -aminoadipoyl)glycine. When this product was heated in 10% (w/v) acetic acid it yielded substances which behaved like cephalosporidine and 6-oxopiperidine-2-carboxylic acid respectively on paper chromatography and electrophoresis.

Since the yields obtained in this process were low, a synthesis on a larger scale was carried out by a method analogous to one described by Rudinger (1954*a, b*) for the synthesis of γ -glutamyl peptides (Professor J. Rudinger kindly informed us of unpublished work in which he had extended the method to peptides of α -aminoadipic acid). DL-N-(Toluene-*p*-sulphonyl)- α -aminoadipic acid was cyclized to DL-1-(toluene-*p*-sulphonyl)-6-oxopiperidine-2-carboxylic acid and the latter allowed to react with aminoacetal to give DL- δ -(N-toluene-*p*-sulphonyl- α -aminoadipoyl)aminoacetaldehyde diethylacetal. Removal of the toluene-*p*-sulphonyl group with sodium in liquid ammonia yielded DL- δ -(α -aminoadipoyl)aminoacetaldehyde diethylacetal. This compound was heated in 10% (v/v) acetic acid at 100° for 1.5 hr., and a crystalline ninhydrin-negative substance which showed no net charge at pH 4.5 was isolated from the reaction products.

The synthetic compound was indistinguishable from cephalosporidine monohydrate in its ultra-violet-absorption spectrum and behaviour on paper chromatography and electrophoresis. But the infrared-absorption spectra of the two crystalline substances, although nearly the same between 2000 and 950 cm^{-1} , showed significant differences between 4000 and 2000 cm^{-1} and between 950 and 700 cm^{-1} . When heated above 190° both substances underwent a change in crystal form, the change being accompanied by sublimation. A sample of each substance was therefore heated in a glass tube at 200–210° in a high vacuum. The resulting crystalline sublimates showed the characteristic ultra-violet-absorption spectrum of cephalosporidine,

gave identical infrared-absorption spectra, and were not distinguishable by m.p. and mixed m.p. Their infrared-absorption spectra were identical with the spectrum obtained from the synthetic compound before sublimation. The sublimate from cephalosporidine, unlike the parent compound, showed no measurable optical activity in water or 6*N*-hydrochloric acid. These results indicated that cephalosporidine and the synthetic DL-compound differed only in optical configuration and that cephalosporidine racemized when heated above 190°.

EXPERIMENTAL

Methods

Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.) and in butan-1-ol-acetic acid-pyridine-water (15:10:3:12, by vol.). Electrophoresis on Whatman no. 1 paper was carried out: (a) for 2.5 hr. at 14 v/cm. in collidine-acetate buffer (0.05*M* with respect to acetate), pH 7.0, and in 10% (v/v) acetic acid, pH 2.2, in an apparatus similar to that of Flynn & de Mayo (1951); (b) for 30 min. at 83 v/cm. in pyridine-acetate buffer (0.05*M* with respect to acetate), pH 4.5, in an apparatus similar to that of Katz, Dreyer & Anfinsen (1959); (c) for 40 min. at 61 v/cm. in (NH₄)₂CO₃ buffer (7.9 g./l.), pH 8.9, as described by Gross (1958, 1961). Cephalosporidine and 6-oxopiperidine-2-carboxylic acid were detected on paper by the method of Rydon & Smith (1952) as used by Trown *et al.* (1962), and amino acids were detected by reaction with ninhydrin. Acetic acid and formic acid were detected after electrophoresis at pH 8.9 by spraying the paper with a solution of bromophenol blue (0.025%) and citric acid (0.1%) in acetone-water (9:1, v/v) as described by Gross (1958).

Electrometric titrations (glass electrode) were carried out in water at 20° with a type TTT1 Automatic Titrator (Radiometer, Copenhagen, Denmark). 1-Imidazolylacetic acid was prepared by the method of Easson & Pyman (1932).

Analyses were by Weiler and Strauss, Oxford.

Cephalosporidine from cephalosporin C and penicillin N

Isolation and properties of cephalosporidine. A solution of the sodium salt of cephalosporin C (2 g.) in water (100 ml.) was heated under reflux for 16 hr. in an oil bath at 130°. Then 12*N*-HCl (22 ml.) was added and the solution heated under reflux for a further 13 hr. The brown solution was evaporated to dryness *in vacuo* in a rotary evaporator. The residue was dissolved in water (2 ml.) and the solution added to a column (1.5 cm. diam. × 20 cm.) of Dowex 50 (200–400 mesh; H⁺ form; X 8) which was then washed with water until the effluent was nearly colourless. The column was broken up, the resin stirred with water (100 ml.), and pyridine added slowly to the mixture until the pH of the solution rose to 7.0. The resin was removed by filtration and the filtrate evaporated to dryness *in vacuo*.

The resulting brown gum was subjected to countercurrent distribution in an all-glass 24-tube micro-apparatus, the volumes of the upper and lower layers being 3 ml. and

1.5 ml. respectively. The solvent system was made by adding 150 ml. of phenol saturated with water to 300 ml. of 5% (v/v) acetic acid saturated with phenol. Fifty transfers were carried out, upper layers being withdrawn from tube 24. Ether (4 ml.) was then mixed with the contents of each tube, and the lower aqueous layers were separated and shaken three times with ether (3 ml.). The resulting aqueous solutions were evaporated to dryness and the weights of the residues determined. A plot of weight against tube no. showed one major band with a maximum at tube 11. Calculation by the method of Williamson & Craig (1947) indicated that the partition coefficient, *K* (i.e. concn. in upper layer/concn. in lower layer), remained almost constant (0.15) from tube 6 to tube 17. The aqueous solutions from these tubes were combined and evaporated to dryness to yield a light-brown gum, most of which crystallized on standing.

This product was dissolved in water (1 ml.) and the solution added to a column (1 cm. diam. × 40 cm.) of Dowex 50 (200–400 mesh; H⁺ form; X 8). Elution was begun with *N*-HCl, 3 ml. fractions being collected every 30 min. After 45 fractions had been collected, elution was continued with 2*N*-HCl. Measurements of extinctions at 211 *mμ* showed that a band of material had been eluted in fractions 41–79 with a maximum in fraction 57. Fractions 47–74 were combined and the resulting solution was evaporated to dryness. The residue was dissolved in water (5 ml.) and this solution added to a column (2 cm. diam. × 5 cm.) of Dowex 1 (200–400 mesh; acetate form; X 8). The column was washed with water (30 ml.) and the effluent evaporated to dryness to yield crystalline cephalosporidine (198 mg.). This product was dissolved in warm ethanol (0.8 ml.) and acetone added dropwise. A small amount of oil which first separated was removed by centrifuging. Further addition of acetone resulted in the separation of cephalosporidine crystals (115 mg.).

Cephalosporidine behaved as a hydrate. On drying at 100° *in vacuo* it lost weight (10.4%) which it regained on exposure to laboratory air. When heated on a Koffler block the hydrate softened or melted and then changed in crystal form above 190°. The resulting mass of highly birefringent flat needles melted at 236–238°. Analyses were made on the hydrated product (Found: C, 51.1; H, 6.3; N, 14.9%; equiv. by titration 180 ± 15; mol. wt. from X-ray crystallographic measurements, by Professor Dorothy Hodgkin and Mr K. Watson, on crystals obtained by allowing an aqueous solution to evaporate in air, 185 ± 5. Calc., from loss of weight on drying, for the anhydrous product: C, 57.1; H, 5.7; N, 16.6%. C₈H₁₀N₂O₂·H₂O requires C, 52.1; H, 6.5; N, 15.2%; loss of weight for 1H₂O, 9.8%; C₈H₁₀N₂O₂ requires C, 57.8; H, 6.0; N, 16.8%). The infrared-absorption spectrum of cephalosporidine monohydrate showed *ν*_{max.} (in KBr) 3550, 3450, 2500 (broad), 1910 (broad), 1605, 1370, 1300, 1240, 1190, 1155, 955, 895, 840, 775, 725 and 692 cm.⁻¹. When chromatographed on paper in butan-1-ol-acetic acid-pyridine-water (15:10:3:12, by vol.) the substance showed *R_F* 0.28.

Formation of cephalosporidine from cephalosporin C (free acid). Dowex 50 (X 8) was added slowly to a stirred solution of the sodium salt of cephalosporin C (20 mg.) in water (1 ml.) until the pH of the solution fell to 2.5. After separation of the resin, the solution was heated under reflux for 3 hr. and was then evaporated to dryness *in vacuo*. The residue was dissolved in water (0.2 ml.) and a

sample (5 μ l.) of this solution was subjected to paper chromatography and electrophoresis. A further sample (0.1 ml.) was mixed with 2N-HCl (0.1 ml.), and the mixture heated at 105° for 18 hr. in a sealed tube and then evaporated to dryness. The residue was dissolved in water (0.1 ml.) and a sample of this solution (5 μ l.) was also subjected to paper chromatography and electrophoresis. Treatment of the papers by the method of Rydon & Smith (1952) revealed that cephalosporidine and 6-oxopiperidine-2-carboxylic acid were present before acid hydrolysis. 6-Oxopiperidine-2-carboxylic acid showed a R_F value 1.9 times that for cephalosporidine in butan-1-ol-acetic acid-water (4:1:4, by vol.) and migrated about 3 times as far as α -aminoadipic acid towards the anode at pH 4.5. After acid hydrolysis cephalosporidine and α -aminoadipic acid were present.

Formation of cephalosporidine from impure penicillin N. A solution of a preparation of the sodium salt of penicillin N (10 mg.; about 25% pure) in water (0.5 ml.) was heated under reflux for 3 hr. One half of the solution was evaporated to dryness *in vacuo*. The other was heated with 2N-HCl (0.25 ml.) at 105° for 18 hr. and then evaporated to dryness. Both residues were dissolved in water (12 μ l.) and samples (5 μ l.) of the resulting solutions were analysed by paper chromatography and electrophoresis. In both cases a compound which was indistinguishable in its behaviour from cephalosporidine was revealed on the paper by the method of Rydon & Smith (1952).

Oxidation with hydrogen peroxide. A solution of cephalosporidine (2.1 mg.) in '100 vol.' (30%, w/v) hydrogen peroxide (0.2 ml.) was kept at room temperature overnight. Water (1 ml.) was added and the solution evaporated to dryness *in vacuo*. The residue was dissolved in water (0.1 ml.) and a sample of the solution (5 μ l.) subjected to paper electrophoresis at pH 4.5 and to paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.). Much unchanged cephalosporidine was detected on the papers by the chlorination method of Rydon & Smith (1952). In addition, a very small amount of a compound was detected which showed a R_F value 1.2 times that for cephalosporidine and gave a grey colour with ninhydrin. A further sample of the solution (10 μ l.) was mixed with 2N-HCl (10 μ l.) and the mixture heated in a sealed tube at 105° for 16 hr. The hydrolysate was evaporated to dryness *in vacuo*, the residue dissolved in water (10 μ l.) and a sample (5 μ l.) of this solution analysed by paper chromatography and electrophoresis. A weak ninhydrin-positive spot was revealed which corresponded to α -aminoadipic acid.

Oxidation with potassium permanganate. A solution of KMnO_4 (20 mg./ml.) was added dropwise at room temperature to a solution of cephalosporidine (9.1 mg.) in water (0.25 ml.). When 0.5 ml. of the KMnO_4 solution had reacted, after approx. 2 hr., the precipitated MnO_2 was removed by centrifuging and washed with a little water. The brown supernatant and washing were evaporated to dryness *in vacuo*, the residue was mixed with water (0.5 ml.) and the mixture was centrifuged. A sample (10 μ l.) of the resulting colourless solution showed no ninhydrin-positive spot on paper. A further sample (0.2 ml.) was mixed with N-H₂SO₄ (0.2 ml.) and the mixture heated in a sealed tube at 105° for 18 hr. The H₂SO₄ was removed from the hydrolysate by the addition of 0.3N-Ba(OH)₂ until the pH rose to 7.0. The mixture was centri-

fuged and the supernatant was evaporated to dryness *in vacuo*. The residue was dissolved in water (0.4 ml.) and samples (5 μ l.) of the solution were analysed by paper chromatography and electrophoresis. The ninhydrin reagent revealed a spot corresponding to α -aminoadipic acid and a smaller spot corresponding to glycine. The intensity of the spot due to α -aminoadipic acid indicated that the latter had been formed from cephalosporidine in about 20% yield. The remainder of the solution was concentrated tenfold and a sample (10 μ l.) of the concentrate subjected to electrophoresis on paper at pH 8.9. This revealed the presence of small amounts of compounds which behaved like formic acid and acetic acid respectively.

Oxidation with bromine water. Bromine water was added dropwise to a solution of cephalosporidine (10 mg.) in water (1 ml.). Reaction with the Br₂ was at first rapid and then became slower. When about 1 ml. of Br₂ water had been added and 1 drop required approx. 1 min. for decolorization, the solution was evaporated to dryness *in vacuo*. The residue was dissolved in water (0.45 ml.). Half of this solution was mixed with 2N-HCl (0.225 ml.) and the mixture heated at 105° in a sealed tube. The hydrolysate was evaporated to dryness and the residue dissolved in water (0.225 ml.). Samples (5 μ l.) of the solutions of the hydrolysed and unhydrolysed product were subjected to paper chromatography and to electrophoresis on paper at pH 4.5. Before hydrolysis the product appeared to contain approximately equal amounts of α -aminoadipic acid, glycine and δ -(α -aminoadipoyl)glycine. After hydrolysis it contained α -aminoadipic acid and glycine. δ -(α -Aminoadipoyl)glycine migrated 0.75 times as far as aspartic acid and 1.2 times as far as glutamic acid towards the anode at pH 4.5.

Synthesis of DL-cephalosporidine

DL-N-Toluene-p-sulphonyl- α -aminoadipic acid. DL- α -Aminoadipic acid (1.932 g.; 12 m-moles) was dissolved in 4N-NaOH (6 ml.; 24 m-moles) and the solution cooled in ice. A solution of toluene-p-sulphonyl chloride (2.280 g.; 12 m-moles) in acetone (6 ml.) and a further 3 ml. of 4N-NaOH were added simultaneously with vigorous stirring. The acetone was removed *in vacuo* and 2N-HCl then added until the pH fell to 2.0, when a white crystalline precipitate was produced. After the mixture had stood for 2 hr. in a bath at -10° the precipitate was filtered off and dried (2.798 g.; yield 66%). It had m.p. 200-206°, raised by recrystallization from aqueous ethanol (colourless prisms) to 212-213° (2.058 g.; yield 49%) (Found: C, 49.8; H, 5.6; N, 4.6; S, 10.2. C₁₃H₁₇NO₆S requires C, 49.5; H, 5.4; N, 4.5; S, 10.2%).

DL-1-Toluene-p-sulphonyl-6-oxopiperidine-2-carboxylic acid. A solution of toluene-p-sulphonyl chloride (1.285 g.; 6.75 m-moles) in pyridine (2 ml.) was added to a solution of DL-N-toluene-p-sulphonyl- α -aminoadipic acid (1.420 g.; 4.5 m-moles) in pyridine (3 ml.) at 0°. The mixture was stirred for 0.5 hr. at 0° and then kept at -10° for 4 hr. On addition of water (10 ml.) to the resulting deep-red solution a small amount of red gum was precipitated. This was removed by filtration and discarded. The addition of 2N-HCl to the filtrate caused a light-pink crystalline material to come out of solution at pH 1.5. After the mixture had stood for 0.5 hr. at -10° the pH had risen to 2.0. Readjustment of the pH to 1.5 was accompanied by the precipitation of more material. The mixture was kept for a

further 0.5 hr. at -10° and the product then filtered off and dried (0.947 g.; yield 70%). Concentration *in vacuo* of the mother liquor (whose pH had been adjusted to 4.5 with pyridine) followed by the careful addition of 2N-HCl produced a further 287 mg. of the same product (total: 1.234 g.; yield 92%). Recrystallization of a sample of the product from ethanol containing about 5% (v/v) of water raised the m.p. from 206–207° to 206.5–207.5° (colourless needles) (Found: C, 52.5; H, 5.1; N, 4.8; S, 10.5. $C_{13}H_{15}NO_5S$ requires C, 52.5; H, 5.1; N, 4.7; S, 10.8%).

DL- δ -(N-Toluene-*p*-sulphonyl- α -aminoadipoyl)aminoacetaldehyde diethylacetal. DL-1-Toluene-*p*-sulphonyl-6-oxopiperidine-2-carboxylic acid (879 mg.; 2.96 m-moles) and aminoacetal (870 mg.; 6.54 m-moles) were dissolved in toluene (4 ml.) and the mixture was refluxed gently for 15 hr. Evaporation of the toluene gave a brown gum to which ice-cold water (10 ml.) was added. The resulting oily solution was extracted with ether (5 ml.). The aqueous layer was transferred to a titration vessel carrying a glass electrode, cooled in ice, and N-HCl added carefully. At pH 4.8, the solution became cloudy and an oil began to precipitate. When the pH had fallen to 4.0, the mixture was extracted twice with $CHCl_3$ (5 ml.). The extracts were combined, dried (over anhydrous Na_2SO_4) and the $CHCl_3$ was evaporated to give a brown gum which crystallized as clusters of colourless needles (671 mg.; yield 53%) of m.p. 118–122°. A sample recrystallized from ethyl acetate had m.p. 118–119° (Found: C, 53.2; H, 7.0; N, 6.5; S, 7.3. $C_{19}H_{30}N_2O_7S$ requires C, 53.0; H, 7.0; N, 6.5; S, 7.45%).

DL- δ -(α -Aminoadipoyl)aminoacetaldehyde diethylacetal. DL- δ -(N-Toluene-*p*-sulphonyl- α -aminoadipoyl)aminoacetaldehyde diethylacetal (584 mg.; 1.45 m-moles) was dissolved in liquid NH_3 (150 ml.) and small pieces of sodium were added with vigorous stirring until a permanent blue colour was obtained. Then NH_4Cl was added until the blue colour disappeared. The NH_3 was allowed to evaporate from the stirred solution and the last traces of free NH_3 were removed *in vacuo* at 40° . The dry white residue was dissolved in water (10 ml.), the solution stirred for 0.5 hr. with about 30 g. (wet wt.) of Amberlite IRC-50 resin (100–200 mesh; NH_4^+ form), and the resin filtered off and washed. The combined filtrate and washings were concentrated *in vacuo* to about 3 ml. and N-Ba(OH) $_2$ was then added until precipitation of $BaSO_4$ and $BaSO_3$ was complete. The solution was filtered, the precipitate washed with water, and the combined washings and filtrate were again concentrated to about 3 ml. *in vacuo*. The resulting solution was added to a column (1 cm. diam. $\times$ 10 cm.) of Amberlite IRC-50 (100–200 mesh; NH_4^+ form) and the column washed with water (50 ml.). The eluate was evaporated to dryness *in vacuo* yielding a white solid (718 mg.; theoretical yield 375 mg.). A portion (580 mg.) of this crude reaction product (which contained relatively large amounts of chloride) was dissolved in 50 ml. of pyridine-acetate buffer (0.05M with respect to acetic acid), pH 5.0, and subjected to electrophoresis in the same buffer in a Beckman Spinco model CP continuous-flow paper-electrophoresis cell. The cell was run at a constant current of 80 mA (approx. 600 v), the sample was fed to the curtain during 24 hr., and 32 fractions were collected. The point of application of the sample was opposite fractions 15 and 16, the fractions being numbered from cathode (1) to anode (32). Ninhydrin-positive material was present in fractions 13–16 and also in 22–24. Evaporation of the latter fractions yielded a residue which was

shown, by paper electrophoresis and chromatography, to contain a relatively small amount of α -aminoadipic acid. Fractions 13–16 were combined and evaporated to dryness *in vacuo* in a rotary evaporator. The resulting white paste was ground with anhydrous acetone (30 ml.) to give a white powder which was separated by centrifuging and dried (163 mg.). The product was not obtained crystalline. It had m.p. 170–172° (Found: C, 50.0; H, 8.8; N, 9.6. $C_{12}H_{24}N_2O_5$ requires C, 52.2; H, 8.7; N, 10.2%). Its infrared-absorption spectrum showed ν_{max} (in Nujol) 3450, 1700, 1610, 1150 and 1070 cm^{-1} . It was ninhydrin-positive and showed no net charge when subjected to electrophoresis on paper at pH 4.5 or 7.0.

DL- δ -(α -Aminoadipoyl)glycine. A sample (2 mg.) of the product from the preceding experiment was dissolved in 0.1N- H_2SO_4 (0.1 ml.) and the solution heated at 100° for 15 min. A solution of 0.3N-Ba(OH) $_2$ was added until no further $BaSO_4$ was precipitated. Freshly precipitated Ag_2O (40 mg.) suspended in water (1 ml.) was added and the mixture was stirred and heated at 80° for 1 hr. and then filtered. The pH of the filtrate was brought to 3.0 with 0.01N-HCl and the precipitated AgCl removed by centrifuging. The supernatant was evaporated to dryness and the residue dissolved in water (11.7 μ l.). Samples (4.5 μ l.) of this solution were subjected to electrophoresis on paper at pH 4.5 and to chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.), samples of authentic D- δ -(α -aminoadipoyl)glycine being used as markers.

DL-Cephalosporidine. δ -(α -Aminoadipoyl)aminoacetaldehyde diethylacetal (150 mg.) was dissolved in 10% (v/v) acetic acid (4.5 ml.) and heated in a sealed Pyrex tube at 100° for 1.5 hr. The resulting solution was applied to a column (2 cm. diam. $\times$ 5 cm.) of Dowex 50 (100–200 mesh; H^+ form; X4) which was then washed with water until the pH of the effluent (which contained 6-oxopiperidine-2-carboxylic acid) rose to 4. The extruded resin was then suspended in a small volume of water and the mixture stirred and titrated to pH 7.0 with N-pyridine. The resin was filtered off and washed with water. Evaporation of the combined filtrate and washings produced a brown gum. The gum was dissolved in water (2 ml.) and the solution applied to a column (2 cm. diam. $\times$ 5 cm.) of Dowex 1 (200–400 mesh; acetate form; X10). The column was then washed with water (100 ml.) and the effluent evaporated to dryness to yield a brown gum which crystallized on standing. The crystalline mass was extracted with warm ethanol (3 ml.) and a small amount of insoluble material removed by centrifuging. The ethanol extract was evaporated to dryness in a rotary evaporator, yielding a light-brown gum (49 mg.) which also crystallized. This product was redissolved in warm ethanol (0.2 ml.) and acetone was added dropwise. Brown non-crystalline material was precipitated and removed by centrifuging. Further addition of acetone resulted in the separation of off-white crystals (29 mg.). On heating (Kofler block), this compound changed to a new crystalline form (highly birefringent flat needles) above 190° . The latter melted at 236–238°. The final melting point was unchanged on admixture with a sample of cephalosporidine (m.p. 236–238°) derived from cephalosporin C. The ultraviolet-absorption spectrum of the synthetic compound showed λ_{max} 211 m μ (log ϵ , 3.77) when measured in water, λ_{max} 212 m μ (log ϵ , 3.77) when measured in N-HCl, and λ_{max} 218 m μ (log ϵ , 3.65) when measured in 0.1N-NaOH. The infrared-absorption spectrum

of the synthetic compound (in KBr) was almost identical with that of cephalosporidine from cephalosporin C between 1900 and 950 cm^{-1} ; it differed from the latter, however, in showing bands at 3350, 2620, 2030, 925, 885, 855, 770 and 702 cm^{-1} . When subjected to electrophoresis on paper at pH 2.3, 4.5, 7.0 and 8.9, and chromatographed on paper in butan-1-ol-acetic acid-water (4:1:4, by vol.) or in butan-1-ol-pyridine-acetic acid-water (15:10:3:12, by vol.), the two compounds were indistinguishable. Both were ninhydrin-negative but were revealed as dark-blue spots on the papers by the chlorination procedure of Rydon & Smith (1952).

SUMMARY

1. When cephalosporin C (sodium salt or free acid) is heated in aqueous solution it yields a degradation product ($\text{C}_8\text{H}_{10}\text{N}_2\text{O}_2$) which contains one carboxyl group and one basic nitrogen atom and gives no colour reaction with ninhydrin. This product has been given the trivial name 'cephalosporidine'. It also appears to be formed from penicillin N.

2. The properties of cephalosporidine, including its stability to hot acid and alkali, its ultraviolet-absorption spectrum, and its oxidative degradation to α -aminoadipic acid and δ -(α -aminoadipoyl)-glycine, indicate that its structure consists of a fused imidazole-piperidine-2-carboxylic acid ring system.

3. It is suggested that δ -(α -aminoadipoyl)-aminoacetaldehyde is an intermediate in the formation of cephalosporidine from cephalosporin C and from penicillin N.

4. DL- δ -(α -Aminoadipoyl)aminoacetaldehyde diethylacetal has been synthesized. On heating in dilute acetic acid it yields DL-cephalosporidine.

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APPENDIX

A Nuclear-Magnetic-Resonance Study of Cephalosporidine and Related Compounds

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On the basis of its physical and chemical properties, cephalosporidine was assigned structure (I) (Abraham & Trown, 1963). Proton-resonance spectra of cephalosporidine and model compounds are discussed in relation to the proposed structure,

and it is concluded that they are consistent with this structure. Spectra of all the compounds studied were obtained with a permanent-magnet spectrometer (Leane, Richards & Schaefer, 1959) at a frequency of 29.913 Mc/sec. The spectrum