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The Structure of Cephalosporin C

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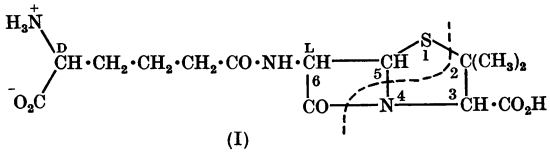
The isolation of cephalosporin C was described by Newton & Abraham (1956). Degradative studies by Abraham & Newton (1956*a*) indicated that cephalosporin C was structurally related to cephalosporin N[(D-4-amino-4-carboxy-*n*-butyl)penicillin; I] (Abraham, Newton & Hale, 1954; Newton & Abraham, 1954; Abraham & Newton, 1954). This paper presents chemical evidence for a definitive structure for cephalosporin C.



(I)

The molecular formula proposed for cephalosporin C ($C_{16}H_{21}O_8N_3S$) had two carbon atoms and two oxygen atoms more than the formula of cephalosporin N (Newton & Abraham, 1956). Cephalosporin C behaved as a monoaminodicarboxylic acid. It contained a residue of D-α-aminoadipic acid, linked to the rest of the molecule through its δ-carboxyl group, and gave 1 mole of carbon dioxide on hydrolysis with hot acid. It yielded L-alanine, valine and glycine, as well as D-α-aminoadipic acid, when hydrolysis was preceded by vigorous hydrogenolysis with Raney nickel. The infrared spectrum of its sodium salt showed a strong band at 5·62 μ, similar to that shown by the penicillins and attributed in the latter to the C=O stretching vibration of the lactam group in the fused β-lactam–thiazolidine ring system (Fig. 1). In these properties cephalosporin C resembled cephalosporin N. Cephalosporin C showed a high degree of resistance to inactivation by penicillinase, but it proved to be a competitive inhibitor of penicillinase (Abraham & Newton, 1956*b*) and an inducer of the synthesis of the enzyme by *Bacillus cereus* and *B. subtilis* (Pollock, 1957).

The close similarities of cephalosporin C and cephalosporin N were accompanied by several striking differences. Cephalosporin C yielded no penicillamine (D-β-mercaptovaline) on hydrolysis and gave 2 moles of ammonia when hydrolysed under conditions in which only 1 mole of ammonia was liberated from cephalosporin N or benzylpenicillin. The valine obtained after treatment of cephalosporin C with Raney nickel was racemic, whereas the valine-yielding fragment of cephalosporin N had the D-configuration. The ultraviolet-absorption spectrum of cephalosporin C sodium salt showed a band with $\lambda_{max.}$ at 260 mμ (log ε 3·95).

We first considered the hypothesis that cephalosporin C contained the skeleton of the characteristic ring system of the penicillins. The infrared-absorption spectrum of cephalosporin C and the ability of the substance to act as a competitive

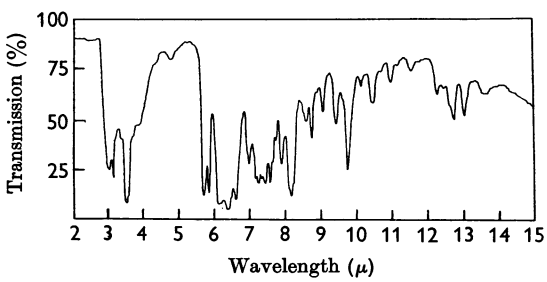


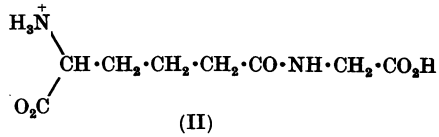
Fig. 1. Infrared-absorption spectrum of cephalosporin C sodium salt (Nujol paste).

inhibitor and an inducer of penicillinase were consistent with this suggestion. It seemed possible to account for the absence of penicillamine from hydrolysates, and for the fact that the valine obtained on hydrogenolysis was racemic, by placing an oxygen function at C-3 in the skeleton of I. A structure of this type also appeared to account for the formation from cephalosporin C, in very small yield, of a substance that resembled penicillaminic acid (β-sulphovaline) in its behaviour on paper electrophoresis at pH 7. This substance was detected after the antibiotic had been subjected to catalytic hydrogenation, the product hydrolysed, and the hydrolysate oxidized with bromine water (Abraham & Newton, 1956*a*). In addition, the structure accommodated the finding that 2 moles of ammonia were liberated on hydrolysis, and that α-oxo*iso*valeric acid

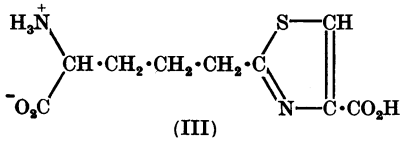
$$[(CH_3)_2CH\cdot CO\cdot CO_2H]$$

was obtained when the antibiotic was treated with Raney nickel at room temperature and the product treated briefly with hot dilute acid (Abraham & Newton, 1958). However, the hypothesis that a modified β-lactam–thiazolidine ring system was present in cephalosporin C threw no light on the nature of the chromophore responsible for the absorption maximum at 260 mμ. The results of more extensive experiments, made possible by the availability of larger amounts of the antibiotic, showed that this hypothesis would have to be abandoned.

Jeffery, Abraham & Newton (1960) reported that δ-amino-δ-carboxyvalerylglycine (II) was formed in small yield when cephalosporin C was hydrolysed with acid and the neutral fraction of the resulting material was oxidized with silver oxide. They also reported that 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (III) was formed when



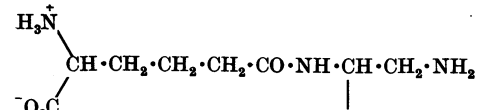
(II)



(III)

cephalosporin C was kept in neutral aqueous solution at 37°. δ-Amino-δ-carboxyvalerylglycine had previously been isolated as a degradation product of cephalosporin N, its formation in this case involving hydrolytic opening of the β-lactam ring, decarboxylation of the resulting potential β-aldehydo acid and oxidation of the potential aldehyde to a carboxylic acid (Newton & Abraham, 1954; Abraham & Newton, 1954). The thiazole (III) contained a sequence of carbon, nitrogen and sulphur atoms identical with that in the fragment of cephalosporin N that appears to the left of the broken line dissecting (I). Its formation was understandable if a similar structural fragment occurred in cephalosporin C and if an elimination reaction resulted in fission of the bond between the sulphur atom of this fragment and the remaining portion of the molecule.

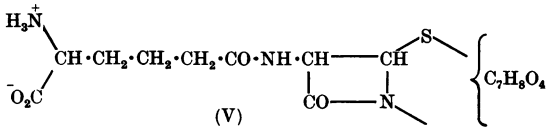
Further evidence for the sequence of atoms near to the side chain of cephalosporin C came from a study of the ninhydrin-positive compounds formed when the antibiotic was treated with Raney nickel at room temperature and the product hydrolysed with N-hydrochloric acid at 100° for 4 min. The resulting mixture was resolved by electrophoresis on paper at pH 7·0 followed by chromatography on paper in butanol–acetic acid–water (4:1:4, by vol.). An acidic component of the mixture corresponded in mobility and R_F with α-aminoadipic acid. Three neutral substances, which gave minor purple spots when coloured with ninhydrin, corresponded with glycine, alanine and valine. A fourth neutral substance gave a major purple spot with R_F 0·054. The latter substance, after elution from the paper, was hydrolysed by further treatment with acid. The hydrolysate contained an acidic compound that behaved like α-aminoadipic acid and a base (R_F 0·09) that gave a grey–brown spot with ninhydrin and migrated towards the cathode at pH 7·0 about 0·8 times as fast as an ornithine marker. The base was indistinguishable, in mobility, R_F and the ninhydrin colour it gave on paper, from an authentic sample of $\alpha\beta$-diaminopropionic acid. It was thus concluded that the substance with R_F 0·054 was the diaminodicarboxylic acid (IV).



(IV)

Consideration of structures II, III and IV, in conjunction with the fact that the infrared-absorption spectrum of cephalosporin C contained bands that could be attributed to the presence of a fused β-lactam ring (5·62 μ) and a monosubstituted amide (6·03 and 6·53 μ), enabled the antibiotic to be assigned, with some confidence, the partial structure (V). This structure contained the skeletons of all three degradation products and could be assumed to give rise to these products by processes that were readily acceptable.

Acceptance of the partial structure (V) left a fragment $C_7H_8O_4$, containing one acidic group, to be accounted for. The formation of DL-valine and of α-oxo*iso*valeric acid on hydrogenolysis of cephalosporin C indicated that five of the seven carbon atoms were associated with the skeleton (VI) or (VII), or possibly a skeleton from which (VII) could be formed by migration of a double bond.



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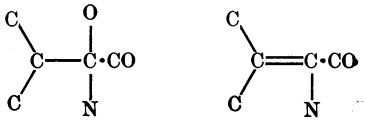


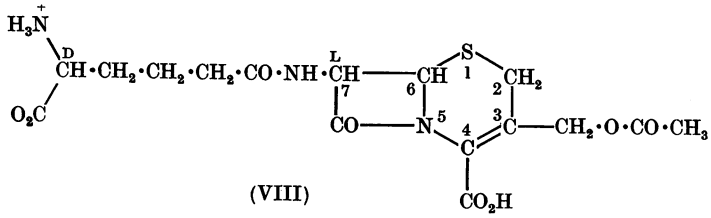
(VI) (VII)

The nature of the remaining C_2 fragment was established when 1 mole of acetic acid was shown to be liberated from cephalosporin C by hydrolysis with 1·25N-sulphuric acid for 1 hr. at 100°, or with N-sodium hydroxide for 1·5 hr. at room temperature. The acetic acid was characterized by the preparation of its *p*-bromophenacyl derivative and by conversion into the corresponding hydroxamic acid and chromatography of the latter on paper. The presence of a band at 5·77 μ in the infrared spectrum of cephalosporin C suggested that the acetic acid was derived from an acetoxyl group. A band at 9·7 μ could then be assigned to the O–C stretching vibration of the $CH_3 \cdot CO–O–C$ grouping.

Cephalosporin C gave approximately one *C*-methyl in the Kuhn–Roth determination, and this was accounted for if an acetoxyl group were present in the molecule. The presence, in addition, of a *gem*-dimethyl group [corresponding to the $(CH_3)_2C$ group of the valine and α-oxo*iso*valeric acid obtained as degradation products] did not appear to be excluded by this finding, since the $(CH_3)_2C$ group may give only a fraction of one equivalent of acetic acid in the oxidative procedure (Kuhn & Roth, 1933). No clear evidence for or against the presence of a $(CH_3)_2C$ group was provided by the infrared spectrum of cephalosporin C sodium salt in Nujol. However, on the assumption that a *gem*-dimethyl group was present, no plausible structure that was consistent with the ultraviolet-absorption spectrum of cephalosporin C could be derived from (VI); and no further development of (VII) could be made, since ozonolysis of cephalosporin C under the conditions recommended by Kuhn & Roth (1932) for the estimation of *iso*propylidene groups indicated that a $(CH_3)_2C:C$ group was absent. Further, attempts to demonstrate the formation of α-oxo-β-thiol*iso*valeric acid on acid hydrolysis of cephalosporin C were unsuccessful. Two crystalline sulphur-containing enolic compounds, which contained no *C*-methyl or nitrogen (subsequently referred to as compounds 1 and 2) were isolated from acid hydrolysates. These compounds appeared to be formed from the C_5 fragment of cephalosporin C that could also give rise to valine, but acceptable structures for them could not be derived from the assumption that the antibiotic C contained sulphur in a five-membered ring.

The question whether a *gem*-dimethyl group was present in cephalosporin C was answered decisively by a nuclear-magnetic-resonance spectrum, for which we were indebted to Mr P. Higham and Dr R. E. Richards. The spectra of the sodium salts of cephalosporin C and benzylpenicillin were determined in aqueous solution, the solvent water being used as an internal standard and the values corrected to the τ scale, on which silicon tetramethyl represents a shift of 10·0 p.p.m. In the spectrum of benzylpenicillin it was possible to account for all the hydrogen present and a large peak at 7·9 p.p.m. was due to the *gem*-dimethyl group. The spectrum of cephalosporin C showed no such peak. A peak of the intensity required for one methyl was present at 7·4 p.p.m., superimposed on a hump due to the combined resonance of the CH_2 and CH groups of the 4-amino-4-carboxy-*n*-butyl side chain. The peak due to this single methyl was in the position expected for the methyl of a $CH_3 \cdot CO \cdot O$ group. Broad resonance at 5·9 p.p.m. could be attributed to isolated CH_2 in or on the ring system, although it was not possible to decide whether one or two such groups were present. There was also an indication of resonance at 4·3 p.p.m., corresponding to the CH–CH resonance in benzylpenicillin.

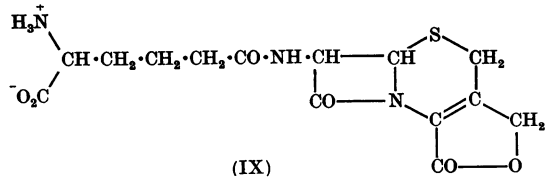
At this stage it occurred to us that the simplest expression of the known properties of cephalosporin C was provided by the structure (VIII), containing a six-membered dihydrothiazine ring. One attraction of this, or a closely related, structure was its ability to account for the formation of a new compound with antibacterial activity, named cephalosporin C_c, when cephalosporin C was kept in 0·1N-hydrochloric acid at room temperature. Cephalosporin C_c showed no net charge when subjected to electrophoresis on paper at pH 5 or pH 7 and Abraham & Newton (1958) had suggested that it was a lactone. Hydrolysis of the acetyl group in (VIII) would lead to a compound from which an αβ-unsaturated-γ-lactone might readily be formed. A second attraction of (VIII) was that it enabled enolic lactone structures (discussed below) to be postulated for compounds 1 and 2.



(VIII)

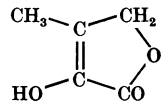
When the structure (VIII) was first considered the direct evidence for it was limited and certain alternatives, in particular a structure with a double bond between C-2 and C-3, were not excluded. But the adoption of (VIII) as a working hypothesis, to be tested by further chemical degradations, soon proved to be fruitful. Before we had made any substantial advance in the subsequent work, Dr Dorothy Hodgkin and Dr E. N. Maslen, who were carrying out an X-ray-crystallographic analysis of cephalosporin C sodium salt, began to discern a sulphur-containing six-membered ring in the molecule. From then on both chemical and X-ray-crystallographical studies made rapid progress. The chemical studies are described below and the X-ray-crystallographical ones by Hodgkin & Maslen (1961).

Cephalosporin C_c was observed to crystallize readily from dilute acetic acid after it had been freed from contaminating acidic products by passage through a column of Dowex 1 (acetate form). Crystalline cephalosporin C_c contained acetic acid of crystallization and the results of elementary analysis indicated that its formula was $C_{14}H_{17}O_6N_3S,CH_3\cdot CO_2H$. It gave only one *C*-methyl in the Kuhn–Roth determination (attributable to acetic acid of crystallization) and hence did not contain the acetoxyl group present in cephalosporin C. It showed an ultraviolet-absorption spectrum similar to that of cephalosporin C, but with $\lambda_{max.}$ at 257 mμ. Its infrared-absorption spectrum showed strong bands at 5·62 and 5·69 μ. It was ninhydrin-positive and yielded α-aminoadipic acid on hydrolysis. It contained an ionizable group with $pK_a < 2{\cdot}4$ and a group with pK_a 9·6. These properties were consistent with the view that cephalosporin C_c was the αβ-unsaturated lactone (IX).



(IX)

Treatment of cephalosporin C or cephalosporin C_c with Raney nickel at room temperature, hydrolysis of the resulting material with acid and extraction of the hydrolysate with ethyl acetate yielded a new crystalline degradation product which was named compound 4. Elementary analysis of compound 4 indicated that a possible molecular formula was $C_5H_6O_3$. The compound contained an acidic group with pK_a 7·7 in water at 20°. It gave a cherry-red colour with ferric chloride in aqueous solution and its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 232 mμ in water (log ϵ 3·9) and $\lambda_{max.}$ at 265 mμ in 0·05N-sodium hydroxide (log ϵ 3·8). These properties suggested that it might be the enolic form (X) of β-methyl-α-oxo-γ-butyrolactone.



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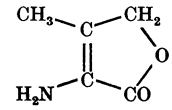
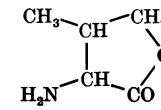


(XI)



(XII)

β-Methyl-α-oxo-γ-butyrolactone was then synthesized by the method of Fleck, Rossi, Hinder & Schinz (1950). The synthetic product, which Fleck *et al.* (1950) had shown to exist in the enolic form, was indistinguishable from compound 4.

A precursor of β-methyl-α-oxo-γ-butyrolactone, named compound 5, was formed when cephalosporin C_c was treated with Raney nickel. Compound 5 was extracted from the aqueous solution with chloroform. It sublimed in a high vacuum at 100°. Its ultraviolet-absorption spectrum in water showed $\lambda_{max.}$ at approximately 250 mμ. When the pH of the solution was lowered from 3 to 1 the band with $\lambda_{max.}$ at 250 mμ was replaced by a new band with $\lambda_{max.}$ at 216 mμ and this change was reversed when the pH was raised again to 3 (Fig. 2). Compound 5 thus appeared to contain a group which was protonated in the pH range 1–3. It migrated as a base when subjected to electrophoresis on paper in 10% (v/v) formic acid and gave a weak-blue spot when the paper was sprayed with ninhydrin. When it was kept in N-hydrochloric acid at 100° for 30 min. its ultraviolet-absorption spectrum changed irreversibly to that of (X).

These properties suggested that compound 5 was the lactone (XI) of αβ-dehydro-γ-hydroxyvaline. The amino group of (XI), linked to an ethylenic carbon which is itself linked to a lactone carbonyl

group, would have only weakly basic properties, and the pK of compound 5 (about 2) appeared to represent a dissociation constant of the right order of magnitude. The ultraviolet-absorption spectra of compound 5 in neutral and acid solution was also consistent with structure (XI). 2-Methylprop-1-ene-1-carboxylic acid lactone would be expected to show $\lambda_{max.}$ at about 216 mμ, since 2-*cyclo*hexylprop-1-ene-1-carboxylic acid lactone shows $\lambda_{max.}$ in this region (Paist, Blout, Uhle & Elderfield, 1941). But the presence of a free amino group as a substituent in an ethylenic system may have a striking bathochromic effect, $\Delta\lambda$ being 40–50 mμ in some cases (see, for example, Braude, 1945). This effect is associated with the presence of an unshared pair of electrons on the nitrogen atom and is abolished when the latter is protonated in acid solution.

Support for structure (XI) was provided by further experiments with the solution obtained by treating cephalosporin C_c with Raney nickel at room temperature. This solution showed an absorption plateau at 240–250 mμ. Hydrogenation of the solution with Adams catalyst resulted in a disappearance of the absorption. Subsequent hydrolysis with N-hydrochloric acid at 100° gave material which was shown by electrophoresis on paper at pH 7 to contain acidic, neutral and basic ninhydrin-positive substances. One of the basic substances, which gave a brownish colour with ninhydrin and migrated towards the cathode 0·44 times as fast as an ornithine marker, corresponded in colour and position to the lactone of authentic γ-hydroxyvaline (XII). This substance, eluted from the paper after electrophoresis, also showed the same R_F as the lactone of γ-hydroxyvaline (R_{Val}, 0·97) on a paper chromatogram run with butanol–acetic acid–water. On boiling with aqueous ammonia, it was converted into a neutral substance with the same R_F (R_{Ala}, 1·05) as that of γ-hydroxyvaline itself. The formation of γ-hydroxyvaline lactone under these conditions was to be expected if cephalosporin C_c yielded a compound with the structure (XI), or this compound together with an N-acyl derivative of it, on desulphurization. The γ-hydroxyvaline was presumably a mixture of stereoisomers.

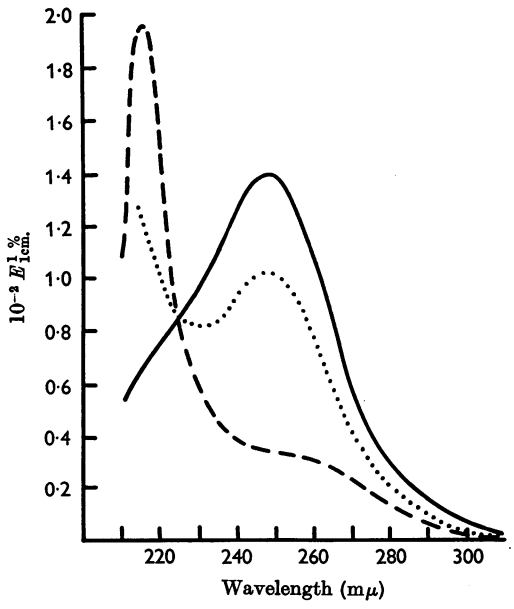


Fig. 2. Ultraviolet-absorption spectrum of compound 5 (not recrystallized). —, In water; ...., in 0·01 N-HCl; – – –, in 0·1 N-HCl.

The identification of the enolic β-methyl-α-oxo-γ-butyrolactone (X) as a degradation product of desulphurized cephalosporin C threw direct light on the nature of compounds 1 and 2 which had been obtained earlier from cephalosporin C itself. When cephalosporin C was heated in 1·25 N-hydrochloric acid at 100° for 1 hr., carbon dioxide was evolved and the absorption band with $\lambda_{max.}$ at 260 mμ disappeared and was replaced by a new band with $\lambda_{max.}$ at 237 mμ. The product responsible for the new absorption band was extracted into ethyl acetate. Although this product crystallized readily from methanol, it was not homogeneous. Chromatography on paper in butanol–acetic acid–water showed that it contained two compounds (1 and 2) with R_F values of 0·81 and 0·85 respectively. When the mixture was subjected to electrophoresis on paper at pH 7·0, compound 1 migrated towards the anode as fast as α-aminoadipic acid and compound 2 migrated more slowly. The two compounds could be revealed on paper by their absorption of ultraviolet light and by the fact that they gave an immediate brown colour when sprayed with aqueous silver nitrate. They were separated on a preparative scale by countercurrent distribution in the system n-butyl acetate–water.

Analytical data showed that a possible formula for compound 1 was $C_{10}H_{10}O_6S$ and that a probable formula for compound 2 was $C_{10}H_{10}O_5S_2$. Neither compound showed any C-methyl in the Kuhn–Roth determination and neither appeared to have optical activity. Compound 1 contained two weakly acidic groups with pK_a values of 6·3 and 9·9 in 50 % (v/v) dimethylformamide. Compound 2 contained two acidic groups with pK_a values of 6·8 and 9·8 in 50 % (v/v) dimethylformamide. The ultraviolet-absorption spectrum of compound 1 showed $\lambda_{max.}$ at 235 mμ in water and $\lambda_{max.}$ at 280 mμ in 0·05 N-sodium hydroxide (Fig. 3). That of compound 2 showed $\lambda_{max.}$ at 243 mμ in water and a broad band between 240 and 330 mμ in 0·05 N-sodium hydroxide (Fig. 4). The infrared spectra of both

compounds showed bands at 3·08 and 5·78 μ which could be attributed to the presence of an OH group and an ester or lactone carbonyl respectively.

Both compounds 1 and 2 gave a strong cherry-red colour with ferric chloride, and gave negative tests with sodium nitroprusside for thiol or disulphide groups. On oxidation with performic acid at $-10°$ they yielded new compounds with R_F values of 0·61 and 0·73 respectively on paper chromatograms run with butanol–acetic acid–water. The new compounds were revealed on paper by their absorption of ultraviolet light and by the fact that they slowly assumed a grey colour when sprayed with aqueous silver nitrate. They were possibly the sulphones of compounds 1 and 2.

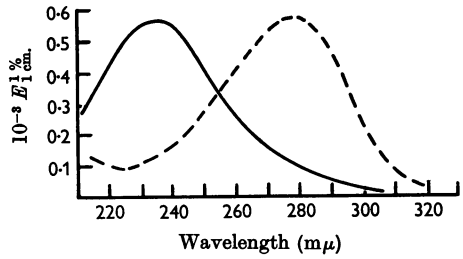


Fig. 3. Ultraviolet-absorption spectrum of compound 1. —, In water; – – –, in 0·05N-NaOH.

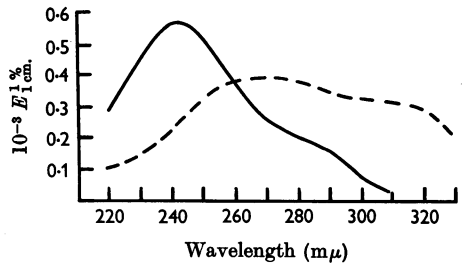
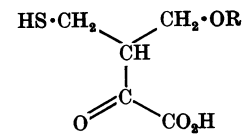


Fig. 4. Ultraviolet-absorption spectrum of compound 2. —, In water; – – –, in 0·05N-NaOH.

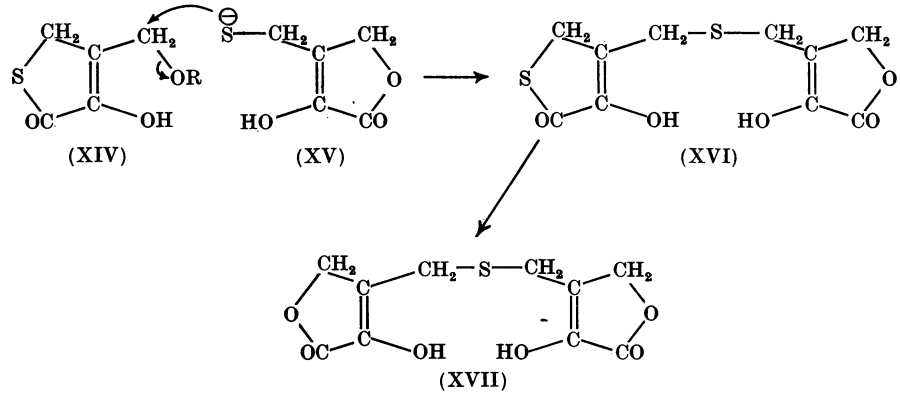
After heating in N-sodium hydroxide at 55° for 10 min. compound 2 gave a strong nitroprusside reaction and a strong smell of hydrogen sulphide was apparent on acidification. After similar treatment compound 1 gave a weak nitroprusside reaction and there was only a faint smell of hydrogen sulphide on acidification. The two compounds also differed in their behaviour when kept in N-hydrochloric acid at 100° for 30 min. Paper chromatography indicated that compound 1 was largely unchanged by this treatment, but that compound 2 was partly converted into compound 1 and a new uncharacterized substance (compound 3) which showed a higher R_F than compound 2 in butanol–acetic acid–water.

Treatment of compound 1 or compound 2 with Raney nickel yielded a product which was indistinguishable from β-methyl-α-oxo-γ-butyrolactone (compound 4) with respect to its ultraviolet-absorption spectrum in water and in 0·05N-sodium hydroxide and was not resolved from the latter when chromatographed on paper in butanol–acetic acid–water or when subjected to electrophoresis on paper at pH 9·0.

The properties of compounds 1 and 2 indicated that they were enolic lactones formed by condensation of two molecules of the C_5 fragment of cephalosporin C. Since they contained no *C*-methyl, but yielded β-methyl-α-oxo-γ-butyrolactone (X) on treatment with Raney nickel, both compounds could be assigned a structure in which the CH_3 group of (X) was replaced by a $-S-CH_2$ group. They would then be derived from a hypothetical intermediate with the structure (XIII), in which R

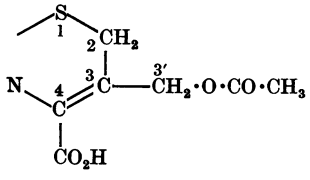


(XIII)



is H or $CH_3 \cdot CO$. The carboxyl group of (XIII) could lactonize with either the thiol group or the potential hydroxyl group to give (XIV) or (XV) respectively. Condensation of (XIV) and (XV), as indicated, would give (XVI), and the latter is suggested as one possible structure for compound 2. $\beta\gamma$-Elimination of sulphur from the ketonic form of the thiolactone ring of (XVI), followed by hydrolytic removal of hydrogen sulphide and relactonization, would yield (XVII). The structure (XVII) is tentatively proposed for compound 1.

The hypothetical intermediate (XIII) in the formation of compounds 1 and 2 could be readily derived from the structure (XVIII). With a view to determining whether cephalosporin C itself contained a double bond in the position of that in (XVIII) the sodium salt of the antibiotic was ozonized in aqueous solution. The solution was then stirred with Raney nickel at room temperature and the resulting volatile material distilled in steam. When the distillate was heated with a solution of 2:4-dinitrophenylhydrazine in 2N-hydrochloric acid an orange-coloured osazone was precipitated in small yield. This osazone was identical with the 2:4-dinitrophenylosazone of authentic hydroxyacetone. Hydroxyacetone is not the only compound from which this osazone can be formed by reaction with 2:4-dinitrophenylhydrazine. However, the isolation of the osazone was consistent with the presence of the grouping (XVIII) in cephalosporin C.



(XVIII)

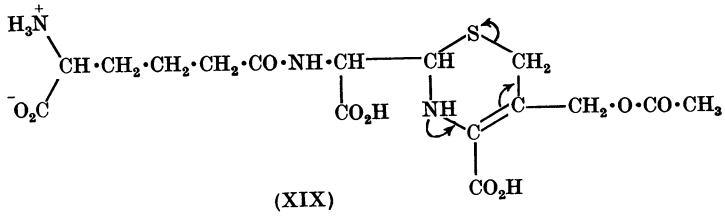
No satisfactory alternative to (XVIII) could be formulated. Thus, a linkage of S to C-3 required that C-2 be joined to C-3′ or C-4 to form a *cyclo*propane ring, since cephalosporin C contained only one *C*-methyl and therefore C-2 could not be a methyl carbon. The resulting structures could account for the formation of valine from cephalosporin C on hydrogenolysis since the *cyclo*propane ring in the amino acid hypoglycin was known to be cleaved by catalytic hydrogenation (Ellington, Hassall & Plimmer, 1958; De Ropp *et al.* 1958; Renner, Jöhl & Stoll, 1958). However, neither of the structures could accommodate a double bond or account for degradation products with the properties of compounds 1 and 2. A linkage of S to C-3′, which also required the presence of a *cyclo*propane ring, failed to account for the retention of sulphur in compounds 1 and 2 since it contained a hemimercaptal grouping. A linkage of S to C-4 was not feasible on either chemical or stereochemical grounds. A modification of (XVIII) in which C-2 and C-3′ were linked to give a *cyclo*propane ring was inconsistent with the presence of a peak due to an isolated CH_2 group in the nuclear-magnetic-resonance spectrum of cephalosporin C. A variant of (XVIII) in which the double bond was between C-2 and C-3 did not account for the isolation of hydroxyacetone 2:4-dinitrophenylosazone from cephalosporin C after ozonolysis and treatment with Raney nickel.

Since cephalosporin C contained only three nitrogen atoms, the nitrogen of (XVIII) was also the β-lactam nitrogen of (V). Combination of (XVIII) and (V) thus led to (VIII). We concluded that the chemical data gave this structure for cephalosporin C a high degree of probability.

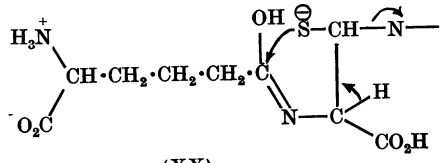
Structure (VIII) accounts for the presence of two strongly acidic groups in cephalosporin C, for the formation of cephalosporin C_c (IX) from cephalosporin C in acid solution at room temperature, for the loss of carbon dioxide in hot acid solution, and for the oxidative degradation of the molecule to δ-amino-δ-carboxyvalerylglycine (II). Reductive removal of the sulphur and the acetoxyl group from (VIII) yields an intermediate from which α-oxo*iso*valeric acid is formed, together with ammonia, on hydrolysis. When hydrogenolysis is accompanied by hydrogenation of a double bond the final product is DL-valine. Hydrolysis of the β-lactam ring of (VIII) leads to (XIX), from the C_5 fragment of which sulphur may be eliminated, as indicated, to form an intermediate (XX) that is converted into the thiazole (III).

Cephalosporin C and cephalosporin C_c show absorption maxima at longer wavelengths (260 and 257 mμ respectively) than would be expected with a normal *N*-acyl αβ-unsaturated amino acid. α-Acetamido-ββ-dimethylacrylic acid was prepared by the method of Abraham, Baker, Chain & Robinson (1949) and showed $\lambda_{max.}$ at 223 mμ. However, in a normal amide the unshared pair of electrons on the nitrogen atom is involved in resonance, whereas in a fused β-lactam ring system there are stereochemical reasons for believing that resonance is suppressed (Woodward, 1949). It may be that suppression of resonance in the β-lactam of cephalosporin C allows the nitrogen of this lactam to exert a considerable bathochromic effect.

When cephalosporin C or cephalosporin C_c was dissolved in 12N-hydrochloric acid at 20° the absorption at 260–257 mμ fell to about 30 % of its original value in 8 min. With cephalosporin C_c the resulting solution showed a strong absorption maximum at 216 mμ and a weak broad maximum in the region of 265 mμ. The maximum at 216 mμ disappeared and that at 265 mμ increased when the



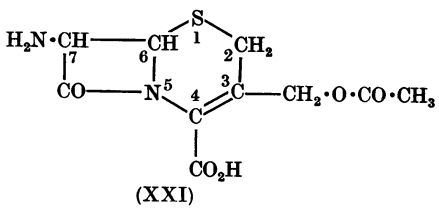
(XIX)



(XX)

pH of the solution was adjusted to 5. Opening of the β-lactam ring in 12N-hydrochloric acid and protonation of the resulting amine nitrogen might be responsible for these changes in absorption.

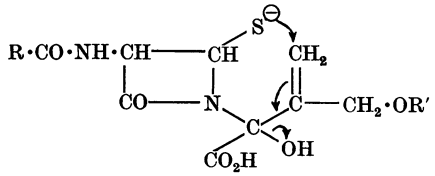
The nucleus of structure (VIII) for cephalosporin C differs from the nucleus of the penicillins in containing a fused β-lactam–dihydrothiazine ring system in place of a fused β-lactam–thiazolidine ring system. The dihydrothiazine ring is a heterocycle that does not appear to have been encountered previously in Nature. The nucleus of the penicillin molecule has been named 6-aminopenicillanic acid (Sheehan, Henery-Logan & Johnson, 1953). We propose the trivial name 7-aminocephalosporanic acid for the nucleus (XXI) of cephalosporin C. Evidence has been obtained that this compound can be produced from cephalosporin C itself (Loder, Newton & Abraham, 1961).



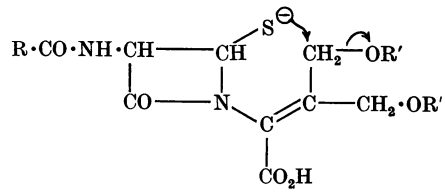
(XXI)

The finding of Arnstein, Morris & Toms (1959) that δ-(α-aminoadipoyl)cysteinylvaline, or the corresponding disulphide, is present in the mycelium of *Penicillium chrysogenum* has raised the possibility that cephalosporin N, or a closely related substance, occupies a special position in the general scheme of penicillin biosynthesis. δ-(α-Aminoadipoyl)cysteinylvaline has a formal resemblance to glutathione and may be synthesized in an analogous manner, by the coupling of α-aminoadipic acid and cysteine, followed by the coupling of the resulting dipepetide with valine. Oxidative cyclization of the tripeptide in the manner postulated by Arnstein & Cawhall (1957), or Birch & Smith (1958), could yield cephalosporin N. Arnstein & Morris (1960) have suggested that benzylpenicillin is formed in *P. chrysogenum* by the action of a transferase on cephalosporin N and that this transferase is lacking in the *Cephalosporium* sp. But it is also possible that the accumulation of cephalosporin N is associated with the fact that the α-aminoadipic acid residue in its side chain has the D-configuration. The *Cephalosporium* sp., but not *P. chrysogenum*, may possess, for example, a racemase by which L-α-aminoadipic acid is converted into the D-isomer at some stage of the biosynthetic process. Arnstein & Morris (1960) isolated α-aminoadipic acid from the mycelium of *P. chrysogenum* and showed that it was mainly the L-enantiomorph.

The similarity in structural pattern of cephalosporin C and cephalosporin N leaves little doubt that the two substances are biogenetically related. The optical configuration at C-7 in cephalosporin C is the same as that at C-6 in the penicillins. Further, X-ray-crystallographic analysis has shown that the hydrogen atoms on the β-lactam ring of cephalosporin C, like those on the β-lactam ring of the penicillins, are *cis* (Hodgkin & Maslen, 1961). It is possible that δ-(α-aminoadipoyl)cysteinylvaline is a common precursor of cephalosporin C, cephalosporin N and benzylpenicillin, and that the biosynthesis of cephalosporin C depends on a mechanism for the oxidation of the *gem*-dimethyl group of the valine residue in the tripeptide. Alternatively, valine may be oxidized before incorporation into the peptide, or an analogue of valine at the appropriate level of oxidation may be formed by a pathway in which valine itself is not an intermediate. Whatever the sequence of events at this stage, structures such as (XXII) or (XXIII) could be envisaged as precursors of the final ring system.



(XXII)



(XXIII)

It is of interest that γ-hydroxyvaline is present in *Kalanchoe daigremontiana* (Pollard, Sondheimer & Steward, 1958) and that a residue of γδ-dihydroxyleucine occurs in the toxic polypeptide phalloidin (Wieland & Schöpf, 1959).

EXPERIMENTAL

Methods

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.); (*B*) butan-1-ol–ethanol–water (5:1:4, by vol.); (*C*) 2-methylbutan-2-ol–propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88) (65:5:30, by vol.) (Isherwood & Cruickshank, 1954); (*D*) butan-1-ol saturated with aq. NH_3 soln. [3 ml. of aq. NH_3 soln. (sp.gr. 0·88) diluted with water to 100 ml.] (Cavallini, Frontali & Toschi, 1949); (*E*) butan-1-ol saturated with 0·1 N-HCl; (*F*) butan-1-ol–ethanol–water (4:1:5, by vol.); (*G*) pentan-1-ol–formic acid–water (3:1:3, by vol.); (*H*) pyridine–ethanol–NH_3 (Herting, Ames & Harris, 1958); (*I*) butan-1-ol–acetic acid–water (4:1:5, by vol.).

Paper electrophoresis on Whatman no. 1 paper (14 v/cm., for 2·5 hr. unless stated otherwise) was carried out as described by Newton & Abraham (1954). The buffers used were: collidine acetate (0·05 M to acetate), pH 7·0; pyridine acetate (0·05 M to acetate), pH 4·5 or 5·0; 10% (v/v) acetic acid, pH 2·2; 10% (v/v) formic acid, pH 1·5; 0·05 M-$(NH_4)_2CO_3$, pH 9·0.

In many cases substances were revealed, after chromatography or electrophoresis on paper, by placing the paper between the viewer and a source of ultraviolet light (230–400 mμ; Corning 9863 filter). Substances which showed strong absorption in the range 240–300 mμ appeared as dark spots.

Electrometric titrations were carried out at 20° in the manner described by Newton & Abraham (1953). Raney nickel was prepared by the method of Pavlic & Adkins (1946) and stored under ethanol at 0°. The amounts of Raney nickel used in different experiments are given as ml. of ethanol-wet product. Antibacterial activities were determined by the hole-plate method, as reported by Abraham & Newton (1956*a*) with *Staphylococcus aureus* or *Salmonella typhi* as the test organism, and cephalosporin C sodium salt as a standard. Analyses were by Weiler and Strauss.

Preparation and paper chromatography of hydroxamic acids. A methanolic solution of hydroxylamine (1·25 N) was prepared by the method of Wingerd (1953), except that the pH was adjusted until the solution gave a green–blue colour on testing with thymol blue.

Samples of aqueous solutions of the acids to be studied (containing 1–5 mg. of each acid) were made alkaline with aq. NH_3 soln. (sp.gr. 0·88) and evaporated to dryness. The salts thus obtained were converted into the corresponding methyl esters by the method of Bergmann & Segal (1956). To the solution of the methyl esters in ether–methanol (about 2 ml.) was added 0·2 ml. of freshly prepared 1·25 N-hydroxylamine. The mixture was kept at 20° for 1 hr. If necessary, 0·1 N-triethylamine in methanol was added drop by drop from time to time until the solution gave a green–blue colour on testing with thymol blue. After 1 hr., 2 μl. of a solution of thymol blue were added to the mixture and the green–blue colour was discharged with CO_2. The solution was then evaporated to dryness in a stream of air and the solid was extracted three times with 50 μl. of dry methanol. The methanolic extracts were evaporated to dryness and the resulting hydroxamic acids dissolved in 50–250 μl. of methanol. Samples (5 μl.) of these solutions were used for paper chromatography. Paper chromatograms of the hydroxamic acids were run in systems *G*, *H* and *I* and the spots were revealed by spraying the papers with 1·0% (w/v) $FeCl_3$ in 95% (v/v) ethanol containing 0·1% of HCl. Formohydroxamic acid showed $R_{\text{acetohydroxamic acid}}$ values in systems *G*, *H* and *I* of 0·73, 0·60 and 0·86 respectively.

Derivatives of cephalosporin C

Cephalosporin C. The antibiotic was used as the crystalline hydrated sodium salt with an activity of 8–10 u./mg. (Newton & Abraham, 1956) (Found: *C*-Me, 3·8; 3·2; acetyl, 10·3. $C_{16}H_{20}O_8N_3SNa,2H_2O$ requires for 1 *C*-Me, 3·2; 1 acetyl 9·0%). A strong band at 8·10 μ in the infrared spectrum of the sodium salt (Fig. 1) could be due to the C–O stretching vibration of an acetoxy group (cf. Jones & Herling, 1956).

Cephalosporin C free acid (Newton & Abraham, 1956) is subsequently referred to as cephalosporin C.

Performic acid oxidation product of cephalosporin C. A solution of performic acid was prepared by adding 30% (w/v) H_2O_2 (0·5 ml.) to AnalaR '98/100%' formic acid (9·5 ml.) and keeping the mixture at 20° for 2 hr. The solution was stored at −10°. Cephalosporin C (80 mg.) was dissolved in formic acid (2 ml.), methanol (0·4 ml.) was added, and the solution cooled to −6°. Cold performic acid solution (6·4 ml.) was then added and the mixture kept at −6° for 2·5 hr. After the addition of 100 ml. of cold water the solution was freeze-dried. The residue was dissolved in water (7 ml.) and the pH of the solution adjusted to 6·4 by addition of 0·1 N-NaOH. The solution was then freeze-dried to give a crude sodium salt (65 mg.). The latter was dissolved in water (0·25 ml.), ethanol (0·25 ml.) was added, and the solution added to a column of Nuchar C (V. A. Howe and Co. Ltd.) (3 cm. × 0·6 cm. diam.). Elution with 50% (v/v) ethanol removed most of the required product in 1 ml. of eluate. Ethanol was added to this eluate until the

mixture remained permanently turbid. The turbidity was removed by centrifuging and ethanol was added drop by drop to the clear supernatant until a further turbidity was seen. The product then separated as a white crystalline sodium salt, which was filtered off and dried in air (28 mg.) (Found: C, 38·1; H, 4·6; N, 8·1; S, 6·5. $C_{16}H_{20}O_9N_3S,2{\cdot}5H_2O$ requires C, 38·5; H, 5·0; N, 8·4; S, 6·4%). The air-dried product showed an activity of 1 u./mg. against *Staph. aureus*. It lost weight (7·1%) corresponding to approximately $2H_2O$ on drying *in vacuo* at room temperature. Its ultraviolet absorption spectrum showed $\lambda_{max.}$ 258 mμ, log ε 3·95. The R_F (0·12) of the oxidation product on paper chromatograms (system *A*) was less than that of cephalosporin C (0·19). When subjected to electrophoresis on paper at pH 7·0, the oxidation product migrated towards the anode at the same rate as cephalosporin C. When lead acetate in 20% (w/v) NaOH was added to a solution of the oxidation product, the solution slowly developed a dark colour, whereas a solution of cephalosporin C immediately gave a heavy chocolate-coloured precipitate (PbS) under similar conditions.

The oxidation product was formed under conditions similar to those reported by Hirs (1956) to oxidize methionine quantitatively to its sulphone. However, the infrared spectrum of the sodium salt of the oxidation product in Nujol was similar to that of cephalosporin C sodium salt. It showed strong bands at 5·66, 5·78, 8·08 and 9·68 μ, but did not show bands near 7·6 and 9·0 μ that would be expected for a sulphone. The band at 9·68 μ in the spectrum of the oxidation product was more intense, relative to the bands at 5·66 and 5·78 μ, than was the band at 9·7 μ, relative to the bands at 5·62 and 5·77 μ, in the spectrum of cephalosporin C. The oxidation product may be the sulphoxide of cephalosporin C. Sulphoxides show an absorption band in the 9·7 μ region (see Bellamy, 1958). The band in the spectrum of cephalosporin C at 9·7 μ may be attributed to the O–C stretching vibration of the $CH_3{\cdot}CO{\cdot}OC$ grouping.

Cephalosporin C_c. Cephalosporin C sodium salt (10 g.) was dissolved in water (150 ml.), and the pH of the solution was adjusted to 2·5 by addition, with stirring, of Dowex 50 X 8 (H form). The mixture was filtered and the resin was washed with about 50 ml. of water. To the filtrate was added AnalaR conc. HCl (25 ml.) and water (65 ml.). The flask containing the acid solution was kept in a water bath at 20° for 18 hr. During this time the extinction of the solution at 260 mμ fell to half its original value. The solution (290 ml.) was then added to a column (46 cm. × 3 cm. diam.) of Dowex 1 X 8 (200–400 mesh; acetate form). After all the solution had entered the column, the latter was washed with water. The effluent was collected in 10 ml. fractions. Neutral material began to emerge from the column in the 18th fraction and by the 45th fraction the extinction of the effluent at 260 mμ had fallen to a low value. The contents of fractions 18–45 were pooled and concentrated to about 30 ml. *in vacuo* in a rotary evaporator. Acetic acid (6 ml.) was added to the concentrate whereupon crystallization of cephalosporin C_c began immediately. After 2 hr. the crystals were filtered, washed with about 3 ml. of ice-cold 3 N-acetic acid, and dried *in vacuo* (yield 3·2 g.). For analysis, a sample of the product (100 mg.) was recrystallized by dissolving it in 1·0 ml. of water and adding 0·18 ml. of acetic acid. After 2 hr. the crystals were filtered and washed with a little ice-cold 3 N-acetic acid (yield 50 mg.).

Cephalosporin C_c showed $[\alpha]_D^{24} + 139°$ in water (*c*, 1·25). It showed an activity of 1 u./mg. against *Staph. aureus* but less than 0·25 u./mg. against *Salm. typhi*. It contained acetic acid of crystallization which was not removed by drying *in vacuo* at 100° (Found: C, 45·9; H, 5·1; N, 9·8; S, 7·5; *C*-Me, 2·2; acetyl, 12·5. $C_{14}H_{17}O_6N_3S,CH_3{\cdot}CO_2H$ requires C, 46·2; H, 5·1; N, 10·1; S, 7·7; 1 *C*-Me, 3·6; 1 acetyl, 10·4%). The ultraviolet-absorption spectrum of the product in water showed $\lambda_{max.}$ 257 mμ, log ε 3·90. The infrared-absorption spectrum (in Nujol) showed strong bands at 3·06, 5·62, 5·69, 6·02 and 6·55 μ.

The nuclear-magnetic-resonance spectrum of cephalosporin C_c was kindly determined by Mr P. Higham and Dr R. E. Richards with a spectrometer operating at 30 Mcyc./sec., the solvent water being used as an internal standard and the values corrected to the τ scale. The spectrum showed an extremely sharp peak at 7·4 p.p.m. This could be assigned to the CH_3 group of the acetic acid of crystallization, since it was intensified, but showed no resolution into more than one component, when free acetic acid (0·5 mole/mole of cephalosporin C_c) was added to the solution. Broad resonance at 5·7 p.p.m. could be attributed to an isolated CH_2 group. The peak at 7·4 p.p.m. was superimposed on a hump due to the combined resonance of the CH_2 and CH groups of the side chain.

The pH of a solution of crystalline cephalosporin C_c (4·9 mg.) in 2 ml. of water was 3·5. Electrometric titration with acid indicated that the substance contained an acidic group with $pK_a < 2{\cdot}4$. On titration with alkali a second acid group (pK_a 4·7, due to acetic acid of crystallization) was detected and a basic group with p*K* 9·6. The equivalent, calculated from the span of the group with pK_a 4·7, was 415 ± 15. Back-titration with acid after the solution had been brought to pH 11·5 and then kept for 1 hr. indicated that a new acidic group had been liberated with pK_a approx. 3·6. The liberation of an acidic group in alkaline solution was associated with a change in the ultraviolet-absorption spectrum, $\lambda_{max.}$ increasing to 265 mμ and log ε at this wavelength falling to 3·65. A further change in the absorption spectrum occurred when a sample of the alkaline hydrolysate was diluted with 50 vol. of 11 N-HCl. The absorption maximum at 265 mμ was replaced by a lower plateau (log ε 3·54) and a new maximum appeared at 216 mμ (log ε 3·90). These changes may be due to opening of the β-lactam ring in alkaline solution and protonation of the liberated NH group of the dihydrothiazine ring in strongly acid solution.

On paper chromatograms, cephalosporin C_c showed R_F 0·1 in system *B* and R_F 0·25 in system *A*. When subjected to electrophoresis on paper at pH 4·5 or 7·0 it behaved as though it had no net charge. At pH 2·2 or 1·5 it migrated towards the cathode at about half the rate of a leucine marker. Spots were revealed by ninhydrin or observation in ultraviolet light.

In aqueous solution at pH 7 cephalosporin C_c was slowly transformed to an acidic ninhydrin-positive substance which migrated towards the anode approximately as fast as cephalosporin C on electrophoresis on paper at pH 7·0. Visual estimation of the intensity of the spots indicated that about half of the cephalosporin C was transformed in 24 hr. under these conditions. The product was indistinguishable in its electrophoretic behaviour from that formed rapidly from cephalosporin C at pH 11·5.

Degradation products of cephalosporin C

αβ-Diaminopropionic acid. Cephalosporin C sodium salt (50 mg.) in water (3·5 ml.) was stirred with Raney nickel (about 0·25 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and washed with about 2 ml. of water. The combined supernatant and washing was extracted four times with 2 ml. of 1% (w/v) 8-hydroxyquinoline in $CHCl_3$ and then three times with 2 ml. of $CHCl_3$. A sample (0·1 ml.) of the aqueous solution was evaporated to dryness *in vacuo*, the residue dissolved in 0·05 ml. of water, and 5 μl. samples of the resulting solution were used for paper chromatography. The main portion of the solution (5 ml.) was acidified with 0·5 ml. of 11 N-HCl and heated at 100°. Samples (0·1 ml.) were removed from the hot mixture, at successive intervals, and evaporated to dryness *in vacuo*. The residues were dissolved in 0·05 ml. of water, and 5 μl. samples used for chromatography and electrophoresis on paper.

Paper chromatography in system *A* showed that the product of hydrogenolysis (before treatment with hot HCl) was a complex mixture. At least seven ninhydrin-positive substances were present, with R_F values between 0·25 and 0·57. After hydrolysis of the product with HCl at 100° for 4 min. the picture was greatly changed. Electrophoresis on paper at pH 7·0 followed by paper chromatography in a second dimension (system *A*) indicated that the mixture contained two major ninhydrin-positive components. One was a neutral substance with R_F 0·054 and the other corresponded in behaviour to α-aminoadipic acid. During hydrolysis with HCl at 100° for 5 hr. the substance with R_F 0·054 gradually disappeared. At the same time the amount of α-aminoadipic acid increased and a new basic substance appeared which gave a grey–brown spot with ninhydrin, showed R_F 0·09, and migrated towards the cathode at pH 7·0 approx. 0·8 times as fast as an ornithine marker (Table 1). The new substance was indistinguishable in its behaviour under these conditions from αβ-diaminopropionic acid.

Liberation of acetic acid. (*a*) On acid hydrolysis. Cephalosporin C sodium salt (250 mg.) was dissolved in 12 ml. of water, 1·8 ml. of 10 N-H_2SO_4 was added, and the solution heated under reflux at 100° for 1·25 hr. The pH of the solution was then adjusted to 2·2 by the addition of 0·3 N-$Ba(OH)_2$. The precipitated $BaSO_4$ was centrifuged down and washed with water, and the combined supernatant and washing was distilled to dryness *in vacuo*. Electrometric titration of the distillate with 0·1 N-NaOH indicated that the latter contained an acid with pK_a 4·8 (0·8 equiv./mole of cephalosporin C). Evaporation of the titrated distillate yielded a crystalline sodium salt. Paper chromatography of the hydroxamic acid obtained from a small sample of this salt (see Methods section) indicated that the latter consisted almost entirely of acetic acid. The main portion of the sodium salt (40 mg.) was dissolved in 0·5 ml. of water, a solution of 100 mg. of *p*-bromophenacyl bromide in 1·0 ml. of ethanol was added, and the mixture was refluxed for 2 hr. After cooling, the crystalline product was filtered off and dried (37 mg.; m.p. 78°). After recrystallization from 1 ml. of aq. 66% (v/v) ethanol it had m.p. 83°. When this product was mixed with authentic *p*-bromophenacyl acetate (m.p. 85°) the m.p. was 84°.

(*b*) On alkaline hydrolysis. Cephalosporin C sodium salt (50 mg.) was dissolved in N-NaOH (1 ml.) and the solution left at 16° for 1 hr. The solution was then added to a column (5 cm. × 1·2 cm. diam.) of Dowex 50X8 (200–400 mesh; H form), and the column washed with water (about 5 ml.). The organic acid in the eluate was converted into the corresponding hydroxamic acid. Paper chromatography indicated that the latter consisted of acetohydroxamic acid together with a trace of formohydroxamic acid.

Hydroxamic acids from other products of hydrolysis. Two uncharacterized products of hydrolysis of cephalosporin C were detected on paper chromatograms in the form of the corresponding hydroxamic acids (referred to as *P* and *Q*). Cephalosporin C sodium salt (25 mg.) was hydrolysed with N-H_2SO_4 (1 ml.) at 100° for 1 hr. The pH was adjusted to 2·0 by addition of 0·3 N-$Ba(OH)_2$ and the precipitated $BaSO_4$ removed. Organic acids in the supernatant were converted into the corresponding hydroxamic acids. The products gave four spots on paper chromatograms. Two spots corresponded with aceto- and formo-hydroxamic acids, the former being about three times as intense as the latter. A third spot (*P*) showed $R_{\text{acetohydroxamic acid}}$ values of 0·4, 0·77 and 0·86 respectively in systems *G*, *H* and *I*. The fourth spot (*Q*) showed $R_{\text{acetohydroxamic acid}}$ values of 0·11, 0·37 and 0·76 respectively. The compounds in the hydrolysate of cephalosporin C which were responsible for the formation of *P* and *Q* were not extractable by ether and did not distil in steam.

Isolation of compounds 1 *and* 2. Crude cephalosporin C sodium salt (2·5 g.; about 65% pure) was dissolved in water (35 ml.) and converted into cephalosporin C by adding sufficient Dowex 50X8 (H form) to the stirred solution to bring the pH, at equilibrium, to 2·7. About 2·5 g. of damp resin was required. The mixture was filtered and the resin was washed on the filter with water. To the clear filtrate (41 ml.), containing cephalosporin C, were added 2 N-HCl (94 ml.) and water (15 ml.) to give 150 ml. of solution which was 1·25 N to HCl. The acid solution was heated for 1 hr. in a flask on a boiling-water bath in a slow stream of N_2. The CO_2 liberated was absorbed in standard NaOH. Back-titration of the NaOH showed that 0·9 mole of CO_2 was evolved per mole of cephalosporin C. The ultraviolet-absorption spectrum of a sample taken from the solution of cephalosporin C immediately after mixing with the 2 N-HCl showed $\lambda_{max.}$ 260 mμ, $E^{1\%}_{1cm.}$ 106. After the solution had been heated for 1 hr. at 100°, it showed $\lambda_{max.}$ 240 mμ and $E^{1\%}_{1cm.}$ 108.

The hydrolysate was cooled and extracted twice with 150 ml. of ethyl acetate. Water (100 ml.) was added to the extract and the mixture was evaporated *in vacuo* until the ethyl acetate had distilled and about 98 ml. of water phase remained. Measurements of ultraviolet-absorption spectra showed that virtually all the material in the hydrolysate with $\lambda_{max.}$ at about 240 mμ had been extracted into the ethyl acetate.

The aqueous solution (95 ml.) obtained from the ethyl acetate extracts was transferred to a smaller flask and evaporated to dryness *in vacuo*, leaving a crystalline mass attached to the walls of the flask by a gum. Cold water (7 ml.) was added, which dissolved most of the gum and detached the crystals. The crystals were filtered off and then dissolved in hot methanol (10 ml.). On concentrating the methanol to about 4 ml. and cooling the solution, crystallization began. After 1 hr. the crystals were filtered and washed with a little ice-cold methanol (yield 130 mg.).

The mother liquor was concentrated to about 1 ml. and yielded a second crop of crystals (40 mg.).

The crystalline product (400 mg., prepared from several batches of cephalosporin C) was subjected to counter-current distribution in the solvent system *n*-butyl acetate–water, 14 ml. of the upper phase and 20 ml. of the lower phase being used in each tube of the machine and the upper phase being mobile. At the start of the experiment the product was loaded equally into tubes 0 and 1. After 100 transfers, combined samples of 0·07 ml. of the upper phase and 0·1 ml. of the lower phase from every second tube were diluted to 3 ml. with ethanol and the extinctions of the resulting solutions measured at 237 mμ. The curve obtained by plotting E against tube no. showed two bands, almost completely resolved, with maxima at tube 65 (E 1·08) and tube 91 (E 1·06). The contents of tubes 52–76 were pooled and evaporated to dryness *in vacuo*, leaving crystalline *compound* 1 (138 mg.), which was recrystallized from about 17 ml. of hot methanol (yield 120 mg.). The contents of tubes 80–95 were pooled and evaporated similarly, leaving crystalline *compound* 2 (202 mg.). This product was recrystallized by dissolving it in about 2·5 ml. of warm methanol and adding 9 ml. of hot water to the solution. After the mixture had cooled the crystals were filtered and washed with a little cold water (yield 173 mg.).

Compound 1. This compound (m.p. 148–149°) crystallized from methanol or water in needles which appeared to lose birefringence on drying. The air-dried crystals (from water) lost 7% of their weight on drying at 80° in a high vacuum. Material dried at 80° was used for analysis (Found: C, 46·1; H, 4·1; S, 11·4; no *C*-Me. $C_{10}H_{10}O_6S$ requires C, 46·3; H, 3·9; S, 12·4%). Compound 1 showed no detectable optical activity in ethyl acetate ($[\alpha]_D^{20} < \pm 3°$). Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 235 mμ ($E^{1\%}_{1cm.}$ 580) in water and $\lambda_{max.}$ 280 mμ ($E^{1\%}_{1cm.}$ 580) in 0·05N-NaOH. Its infrared-absorption spectrum (in Nujol) showed strong bands at 3·08 μ (OH), 5·76 μ (lactone C=O), 5·90 μ (C=C?), 8·78 μ and 12·88 μ. Its equiv. [by electrometric titration in 50% (v/v) dimethylformamide] was 135±10. An aqueous solution of compound 1 gave no precipitate on the addition of 2:4-dinitrophenylhydrazine in 2N-HCl.

Dimethyl ether of compound 1. When compound 1 (10 mg.) in methanol (0·2 ml.) was treated with a small excess of ethereal diazomethane it was converted into a new product (presumably the dimethyl ether) which crystallized on evaporation of the solvent. After recrystallization from $CHCl_3$–di*iso*propyl ether the new product (6·2 mg.) had m.p. 71–72°. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 230 mμ both in ethanol and in 0·05N-NaOH in 95% (v/v) ethanol. In the latter solvent an absorption band with $\lambda_{max.}$ at 278 mμ appeared after 20 hr. at 20°, presumably due to hydrolysis of an OMe group. The infrared spectrum of the dimethyl ether (in $CHCl_3$) showed strong bands at 5·70 μ (lactone C=O) and 5·95 μ (C=C?).

Desulphurization of compound 1. Raney nickel (about 0·1 ml.) was added to a solution of compound 1 (10 mg.) in water (1 ml.) and 0·1N-NaOH (0·35 ml.). The mixture (pH 7) was stirred for 15 hr. at room temperature. The Raney nickel was removed by centrifuging and the supernatant, after adjustment to pH 4, shaken twice with 1 vol. of ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and the solvent then removed in a stream of N_2. The crystalline residue (3 mg.) was sublimed at 45° and 0·07 mm. The sublimate was indistinguishable from compound 4 (α-oxo-β-methyl-γ-butyrolactone) in m.p. (88°), ultraviolet-absorption spectrum in water and in 0·05N-NaOH, rate of migration towards the anode on paper electrophoresis at pH 9·0 and R_F on paper chromatography in system *A*.

Compound 2. The air-dried compound (m.p. 178–179°) was dried at 80° in a high vacuum (loss of wt. 6·7%) for analysis. (Found: C, 43·4; H, 3·8; S, 23·1; no *C*-Me. $C_{10}H_{10}O_5S_2$ requires C, 43·8; H, 3·7; S, 23·3%). The compound showed no detectable optical activity. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 234 mμ ($E^{1\%}_{1cm.}$ 580) in water and a broad band between 240 and 330 mμ ($E^{1\%}_{1cm.}$ 400 at 265 mμ) in 0·05N-NaOH. Its infrared spectrum (in Nujol) showed strong bands at 3·05 (OH), 5·77 μ (lactone C=O?), 5·90 μ (C=C?), 5·97 μ (thiolactone C=O?), 6·12 μ, 8·75 μ and 9·70 μ. Its equiv. [by electrometric titration in 50% (v/v) dimethylformamide] was 140±10. An aqueous solution of compound 2 gave no precipitate on addition of 2:4-dinitrophenylhydrazine in 2N-HCl.

Desulphurization of compound 2. A neutral aqueous solution of compound 2 (20 mg.) was stirred with Raney nickel under the conditions described for compound 1. A portion (3·5 ml.) of the resulting solution (total vol. 4·5 ml.) was extracted with ethyl acetate and yielded a crystalline product (2·0 mg.) which was indistinguishable from authentic α-oxo-β-methyl-γ-butyrolactone (compound 4). To the remainder of the solution (1 ml.) was added $NaIO_4$ (10 mg.). The mixture was kept at 20° for 2 hr. and freeze-dried, and 2:4-dinitrophenylhydrazine in 2N-HCl was added to the distillate. Orange–yellow 2:4-dinitrophenylhydrazone precipitated (2 mg.), which was partly crystalline. On paper chromatograms run under the conditions described by Lynn, Steele & Staple (1956) this product yielded three yellow spots corresponding in R_F values with the 2:4-dinitrophenylhydrazones of formaldehyde, acetaldehyde and *iso*butyraldehyde. *iso*Butyraldehyde and formaldehyde might have been formed by periodate oxidation of the diol $(CH_3)_2{\cdot}CH{\cdot}CH(OH){\cdot}CH_2{\cdot}OH$.

Oxidation of compounds 1 *and* 2 *with performic acid.* A sample (2 mg.) of compound 1 was dissolved in 0·05 ml. of AnalaR formic acid and 0·01 ml. of methanol, and the solution cooled to −10°. Performic acid solution (0·16 ml., prepared as described earlier) was added and the mixture kept at −10° for 2 hr. Water (1 ml.) was added and the solution freeze-dried. The residue was dissolved in 0·2 ml. of water. Compound 2 was oxidized in a similar manner. Samples (5 μl.) of the resulting solution were used for paper chromatography in system *A*. Detection of spots by their absorption of ultraviolet light showed that compound 1 had R_F 0·77 and compound 2 had R_F 0·84, whereas the R_F values of the corresponding oxidized compounds were 0·66 and 0·73 respectively. When the paper was sprayed with 1% (w/v) $AgNO_3$, compounds 1 and 2 were revealed immediately as light-brown spots, whereas the corresponding oxidized compounds gave grey spots which appeared after about 30 min.

Effect of hydrochloric acid at 100° *on compounds* 1 *and* 2. Samples (0·8 mg.) of compounds 1 and 2 in 1·25N-HCl (0·2 ml.) were heated in sealed tubes at 100° for 1 hr., and 0·5 μl. samples of the resulting solutions (1 and 2 respectively) were analysed by paper chromatography and paper electrophoresis. Solution 1 showed only a single spot in-

distinguishable from that shown by the original compound 1. Solution 2 showed a spot in the position occupied by the original compound 2 (R_F 0·84 in system *A*; migration 4·7 cm. towards the anode at pH 7·0) and two further spots, the first corresponding to compound 1 (R_F 0·77 in system *A*; migration 7·0 cm. towards the anode at pH 7·0) and the second to a new product, compound 3 (migration 2·0 cm. towards the anode at pH 7·0). The $R_{\text{compound 2}}$ values of compounds 1 and 3 in system *E* were 0·8 and 1·1 respectively. Compound 3, like 1 and 2, was revealed on paper by its absorption of ultraviolet light and the fact that it gave a brown colour when sprayed with aq. $AgNO_3$. Compounds 2 and 3 gave a red colour when the paper was sprayed with a 0·1% solution of 2:3:5-triphenyltetrazolium chloride in butan-1-ol saturated with water, dried, and then sprayed with 10N-NaOH–ethanol–butan-1-ol (1:4:5, by vol.). Compound 1 showed no colour under these conditions.

Synthesis of β-mercapto-α-oxoisovaleric acid 2:4-dinitrophenylhydrazone. An attempt to remove the benzyl group from the *S*-benzyl derivative of β-mercapto-α-oxo*iso*valeric acid (Hems, Holland & Robinson, 1949) with Na in liquid NH_3 was unsuccessful. Desulphurization occurred and the product was α-oxo*iso*valeric acid.

β-Bromo-α-oxo*iso*valeric acid (2·5 g.) (Hems *et al.* 1949) in water (8 ml.) was neutralized by the addition of solid $(NH_4)_2CO_3$, and the solution added slowly to a solution of aq. N-NH_3 soln. which had been saturated with H_2S at 0°. The mixture was kept for 15 min. at room temperature, and ethanol (4 vol.) was then added. No product separated from this solution when it was kept overnight (cf. the synthesis of β-mercaptopyruvic acid by Parrod, 1947). The solution was then adjusted to pH 3 with 6N-HCl, evacuated to remove H_2S, and 2:4-dinitrophenylhydrazine in 2N-HCl added until no further hydrazone was precipitated. *β-Mercapto-α-oxoisovaleric acid 2:4-dinitrophenylhydrazone* crystallized in fine plates (m.p. 143–144°, decomp.) from aqueous ethanol (Found: C, 38·3; H, 4·1; S, 8·8. $C_{11}H_{12}O_6N_4S,H_2O$ requires C, 38·2; H, 4·6; S, 9·2%). The product lost weight (5·2%) corresponding to 1 H_2O on drying *in vacuo* at 100°. It dissolved readily in aq. N-NH_3 soln. and the solution became deep red on addition of sodium nitroprusside. A sample (1·6 mg.) was dissolved in 1 ml. of ethanol, 20 ml. of water was added, and the solution hydrogenated for 24 hr. in the presence of 50 mg. of Adams catalyst (L. Light and Co. Ltd.) (cf. Meister & Abendschein, 1956). The catalyst was removed by filtration, the filtrate evaporated to dryness, and the residue dissolved in 0·1 ml. of water. A sample (5 μl.) of the resulting solution gave a strong spot corresponding to valine when chromatographed on paper in system *A*.

The 2:4-dinitrophenylhydrazone of β-mercapto-α-oxo-*iso*valeric acid behaved similarly to that of α-oxo*iso*valeric acid when subjected to paper electrophoresis at pH 7·0 (migration 2·5 cm. towards the anode) or to paper chromatography in system *C* (R_F 0·71), or system *D* (R_F 0·80). When samples (5 μl.) of solutions of these compounds were mixed with 30% (w/v) H_2O_2 (2 μl.) immediately before application to the paper, the 2:4-dinitrophenylhydrazone of α-oxo*iso*valeric acid appeared to be unchanged, whereas that of β-mercapto-α-oxo*iso*valeric acid was partly converted into a new compound (presumably the sulphonic acid) which migrated 5·0 cm. towards the anode at pH 7·0.

2:4-Dinitrophenylhydrazones of products of hydrolysis of cephalosporin C. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl to the solution (7 ml.) obtained by washing the crude mixture of compounds 1 and 2 with water (see above) yielded a heavy orange-coloured amorphous precipitate. The crude 2:4-dinitrophenylhydrazone contained S and most of it was acidic, being soluble in water at pH 7 and reprecipitated on addition of acid (Found: S, 10·25%) (cf. Abraham & Newton, 1958). On catalytic hydrogenation with Adams catalyst, as described above, it yielded a small amount of an amino acid that behaved like valine on paper chromatography in system *A*. However, on paper chromatography in systems *C* and *D* it formed an almost continuous yellow streak from the origin and showed no spot in a position corresponding to the 2:4-dinitrophenylhydrazone of β-mercapto-α-oxo*iso*valeric acid.

Amorphous precipitates were also formed when 2:4-dinitrophenylhydrazone in 2N-HCl was added to alkaline hydrolysates of cephalosporin C (0·2N-NaOH for 4 hr. at 20°), or of compounds 1 and 2 (N-NaOH for 10 min. at 55°) or to an aqueous solution of cephalosporin C (pH 7) which had been kept at 37° for 48 hr. All these precipitates formed streaks on paper chromatography in systems *C* and *D* and attempts to isolate a crystalline product from them were unsuccessful.

Isolation of α-oxoisovaleric acid 2:4-dinitrophenylhydrazone. A solution of cephalosporin C sodium salt (50 mg.) in 3·5 ml. of water was stirred with Raney nickel (about 0·2 ml.) for 20 hr. at 20°. The Raney nickel was filtered and washed with 1·5 ml. of water. To the combined filtrate and washing was added 2·5 ml. of 3N-HCl, and the solution heated in a boiling-water bath for 4 min. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl (about 2 ml.) to the cooled solution resulted in the rapid formation of an orange crystalline precipitate (4 mg.). After crystallization from hot water this product had m.p. 194–196°. The m.p. was not depressed on admixture with authentic α-oxo*iso*-valeric acid 2:4-dinitrophenylhydrazone (m.p. 194–196°). The two products were indistinguishable on paper chromatograms run in system *C* or *D* and when subjected to electrophoresis at pH 7·0. X-ray powder photographs of the two products were kindly taken by Miss Joan Shipley and Dr Dorothy Hodgkin and reported to be identical.

Isolation of compound 4. (*a*) A solution of cephalosporin C sodium salt (200 mg.) in water (14 ml.) was stirred with Raney nickel (about 1 ml.) for 18 hr. The Raney nickel was removed by centrifuging and washed with 5 ml. of water. The ultraviolet-absorption spectrum of the combined supernatant and washing showed a continuous rise in extinction from 290 to 220 mμ. Hydrochloric acid (0·1 vol., 12N) was added and the resulting solution kept at 100° for 20 min. During this treatment the extinction fell at 290–260 mμ and rose to a maximum at 232 mμ. The solution was then shaken twice with 1 vol. of ethyl acetate. The combined extracts were dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue (10 mg.) consisted of a crystalline product contaminated with a brown oil. On sublimation at 60° and 0·07 mm. it yielded a white crystalline sample of compound 4, m.p. 86–90° (5·5 mg.).

(*b*) To a solution of cephalosporin C sodium salt (1 g.) in water (20 ml.) was added 6·35 ml. of 1·04N-HCl. The solution was kept at 20° for 4 days and its pH then adjusted to 5·5 by the addition of 0·2N-NaOH (26 ml.). Raney nickel (about 5 ml.) was added and the mixture stirred for 18 hr. The Raney nickel was removed by centrifuging and washed with 10 ml. of water. The combined supernatant

and washing was shaken twice with 1 vol. of $CHCl_3$. The solvent was evaporated *in vacuo*, leaving a mixture of crystals and thick oil (65 mg.). Sublimation of the mixture at 100° and 0·07 mm. gave a white crystalline product whose ultraviolet-absorption spectrum in water and dilute HCl was that of compound 5 (Fig. 2). To the aqueous phase remaining after the extraction with $CHCl_3$ was added 0·1 vol. of 12N-HCl. The solution was heated at 100° for 20 min., cooled, and shaken twice with 1 vol. of ethyl acetate. The extract was dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue yielded a white crystalline sample (40 mg.) of compound 4, m.p. 88–90°, on sublimation at 60° and 0·07 mm. (Found: C, 53·2; H, 5·6%; equiv. 115±5. $C_5H_6O_3$ requires C, 52·6; H, 5·3%; equiv. 114.).

Compound 4 could be detected on paper in 50 μg. quantities as an ultraviolet-light-absorbing spot which became more intense after the paper had been sprayed with 0·01N-NaOH, or as a brown spot after the paper had been sprayed with ammoniacal $AgNO_3$ and then heated for 3 min. at 100°. On paper chromatograms run in system *A*, it showed R_F 0·88. When subjected to electrophoresis on paper it showed no significant migration at pH 6·8 but migrated towards the anode at pH 9·0. In all these properties it was indistinguishable from β-methyl-α-oxo-γ-butyrolactone (m.p. 87–89°), synthesized by the method of Fleck *et al.* (1950). X-ray powder photographs of the synthetic product and compound 4 were taken by Dr Dorothy Hodgkin and Mr R. Diamond and reported to be identical. Both crystalline products were unstable, undergoing detectable decomposition with the formation of oily material within 2 days (cf. Fleck *et al.* 1950).

Ozonolysis of cephalosporin C. A solution of cephalosporin C sodium salt (20 mg.) in water (3 ml.) was cooled to 0°, and ozonized O_2 (containing about 6% of O_3) passed through the solution. Ozonization was continued for 15 min. after O_3 appeared in the exit gas (test with KI); N-$KMnO_4$ (3 ml.) was then added and the mixture heated under reflux for 7 min. (cf. Kuhn & Roth, 1932). The resulting solution was distilled from a Markham still, and the distillate collected in 2:4-dinitrophenylhydrazine in 2N-HCl. No precipitation of 2:4-dinitrophenylhydrazone occurred. It was concluded that the *iso*propylidene grouping was not present in cephalosporin C.

Ozonolysis followed by desulphurization with Raney nickel. Ozonized O_2 (containing about 6% of O_3) was passed through a solution of cephalosporin C sodium salt (50 mg.) in water (3·5 ml.) at 0° for 1 hr. The solution was then stirred with Raney nickel (about 0·2 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and the supernatant distilled in steam, successive 12 ml. portions of distillate being collected in tubes containing 1 ml. samples of a solution of 2:4-dinitrophenylhydrazine in 2N-HCl. The solutions in tubes 1 and 2 became heavily opalescent and that in tube 3 faintly opalescent. The contents of tubes 1–4 were combined and heated at 100° for 30 min., when a flocculent orange-coloured osazone precipitated. The osazone was filtered, washed with water and dried *in vacuo* (2 mg.). It crystallized from warm pyridine in needles, m.p. 308–310°.

One drop of authentic hydroxyacetone [60% (w/w) solution in methanol from L. Light and Co. Ltd.] was added to 4 ml. of water, the mixture steam-distilled, and a 2:4-dinitrophenylosazone prepared from the distillate in the manner described for the product from cephalosporin C. Authentic hydroxyacetone 2:4-dinitrophenylosazone showed m.p. 307–310° (cf. Bülow & Seidel, 1924). On admixture with the osazone from cephalosporin C the m.p. was 307–310°. X-ray powder photographs of the two products were kindly taken by Dr E. N. Maslen and reported to be identical.

Desulphurization followed by ozonization. Cephalosporin C sodium salt (75 mg.) in water (5 ml.) was stirred with Raney nickel (about 0·5 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and washed with 1 ml. of water. The combined supernatant and washing was shaken four times with 2 ml. of 1% (w/v) 8-hydroxyquinoline in $CHCl_3$ and then three times with 2 ml. of $CHCl_3$. Ozonized O_2 (6% of O_3) was passed through the clear aqueous solution at 0° for 1 hr. The solution was then distilled in steam and the distillate collected in a solution of 2:4-dinitrophenylhydrazine in 2N-HCl until no further precipitation of 2:4-dinitrophenylhydrazone occurred. The hydrazone was centrifuged down, washed with water and dried (7·2 mg.). It was dissolved in $CHCl_3$ (1 ml.) and added to a column (15 cm. × 1 cm. diam.) packed with a slurry made by mixing 14 g. of Bentonite (Hopkin and Williams Ltd.) and 3·5 g. of kieselguhr as described by Elvidge & Whalley (1955). On elution with $CHCl_3$, part of the crude hydrazone moved down the column as a yellow band. Evaporation of the eluate yielded 4·0 mg. of a crystalline orange–yellow product which had m.p. 158–166° after recrystallization from 95% (v/v) ethanol. On admixture with authentic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 168°) the m.p. was 162–168°. Subsequent elution of the column with $CHCl_3$ containing 3% (v/v) of ethanol removed a second and light-yellow hydrazone (1·3 mg.). The latter had m.p. 154–157°. On admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone (m.p. 162–164°) the m.p. was 154–158°. The first hydrazone was indistinguishable from acetaldehyde 2:4-dinitrophenylhydrazone and the second from formaldehyde 2:4-dinitrophenylhyrazone when chromatographed in heptane saturated with phenoxyethanol on Whatman no. 7 paper treated with 10% (v/v) phenoxyethanol in acetone as described by Lynn, Steele & Staple (1956).

The failure to detect the formation of acetone on ozonolysis of Raney-nickel-treated cephalosporin C indicated that the treatment with Raney nickel did not yield a product which contained the group $(CH_3)_2C{=}C$. However, treatment with Raney nickel followed by brief hydrolysis with acid resulted in the production of some α-oxo*iso*valeric acid. The latter may therefore have been derived from a group such as $H_2C{:}C(CH_3)\cdot CH(N)\cdot CO_2H$, formed from cephalosporin C by hydrogenolysis and migration of a double bond. The small amount of formaldehyde formed when ozonolysis was carried out after hydrogenolysis might have come from a structure of this type. The origin of the small amount of acetaldehyde obtained under these conditions is uncertain. Conceivably it was formed by degradation of an unstable keto acid which remained when a terminal methylene group was removed as formaldehyde.

Degradation products of cephalosporin C_c

Hydrogenolysis with Raney nickel. To 50 mg. of crystalline cephalosporin C_c was added water (3·5 ml.) and Raney nickel (0·36 ml.). The mixture was stirred at 15° for 18 hr.

Most of the Raney nickel was then removed by centrifuging and the supernatant (pH 6·6) was filtered through Whatman no. 42 paper. The precipitated Raney nickel was washed first with water (the clarified washing being added to the filtrate) and then with $CHCl_3$.

Compound 5. The combined filtrate and washing referred to above was extracted three times with 1 vol. of $CHCl_3$, the $CHCl_3$ washing of the Raney nickel being used for the first extract. The extracts were dried over Na_2SO_4 and evaporated to dryness in a stream of N_2, giving feathery crystals of compound 5 (3·6 mg.). The unrecrystallized product showed m.p. 70–75°, $\lambda_{max.}$ 248 mμ, $E^{1\%}_{1cm.}$ 140. The crystalline material was unstable in air but appeared to be stable in a dilute solution in benzene frozen at −20°.

Compound 5 migrated about 0·87 times as fast as isoleucine towards the cathode when subjected to electrophoresis on paper at pH 1·5, but at pH 2·3 it migrated only 0·33 times as fast as isoleucine. Compound 5 could be detected as a dark spot by viewing the papers in ultraviolet light.

Desulphurized cephalosporin C_c. The aqueous solution, which remained after the removal of compound 5 with $CHCl_3$, was extracted three times with 1 vol. of 1% (w/v) oxine in $CHCl_3$, and then freed from oxine by three extractions with 1 vol. of $CHCl_3$. Evaporation of the aqueous layer *in vacuo* left 17 mg. of a crude product (referred to as desulphurized cephalosporin C_c) whose ultraviolet-absorption spectrum showed a marked inflexion at 240 mμ with $E^{1\%}_{1cm.}$ 156. When subjected to electrophoresis on paper at pH 4·5 or 1·5, this product was indistinguishable from cephalosporin C_c. When chromatographed on paper in system *A* it showed a major spot (coloured with ninhydrin) with R_{Ala} 0·92 and a minor spot with R_{Ala} 0·74. One of these spots may have been due to desthiocephalosporin C_c. R_{Ala} of cephalosporin C_c in system *A* was 0·61.

Compound 4 (*1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone*). Desulphurized cephalosporin C_c (16 mg.) was heated with 0·7 ml. of 1·3 N-HCl at 100° for 1 hr. The ultraviolet-absorption spectrum of the hydrolysate now showed $\lambda_{max.}$ at 233 mμ, $E^{1\%}_{1cm.}$ 201. The cooled hydrolysate was extracted three times with 0·5 vol. of ethyl acetate. The extract was dried over $CaCl_2$ and evaporated to dryness in a stream of N_2, giving crystals of compound 4 (4 mg.). This product underwent partial decomposition when kept in a desiccator for several days, but after recrystallization from a mixture of benzene and light petroleum (1:1, v/v) it appeared to be considerably more stable. When the product was subjected to paper chromatography in system *A* and to electrophoresis on paper at pH 9·0, its behaviour was indistinguishable from that of authentic 1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone.

αβ-Diaminopropionic acid. The aqueous residue which remained after extraction of compound 4 from the acid hydrolysate of desulphurized cephalosporin C_c was studied by paper chromatography in system *A*. The chromatogram showed three ninhydrin-positive spots. Spot 1 had R_F 0·054 and spot 2 had the same R_F (0·11) as αβ-diaminopropionic acid. The third spot was not distinguished in R_F from a mixture of alanine and α-aminoadipic acid (Table 1). When the hydrolysate was subjected to electrophoresis on paper at pH 7·0 it was resolved into five ninhydrin-positive spots. One was due to an acidic substance that behaved like α-aminoadipic acid and one to material that showed no net charge. The three remaining spots, which were of minor intensity (spots 2, 4 and 5, Table 1), were due to basic substances. The substance responsible for spot 2 was not resolved from an authentic sample of αβ-diaminopropionic acid and gave the same grey–brown colour as the latter with ninhydrin.

The aqueous residue was hydrolysed further with 6 N-HCl at 110° for 6 hr. and the product again studied by chromatography and electrophoresis on paper. Spot 1 had disappeared whereas spot 2 had become of major intensity and the spot due to α-aminoadipic acid had also increased in intensity. Spots 4 and 5 had undergone little change. Desulphurized cephalosporin C_c, unlike the material obtained by treatment of cephalosporin C with Raney nickel, yielded no detectable amount of valine on hydrolysis.

γ-Hydroxyvaline. Spot 4 (referred to above) did not appear when the hydrolysate was freed from HCl by evaporation *in vacuo* and the residue boiled with aq. 2 N-NH_3 soln. for 5 min. before being subjected to electrophoresis on paper. This property suggested that it might be due to γ-hydroxyvaline lactone. However, since substantial amounts of compounds 4 and 5 had been found in the hydrolysate, only a relatively small yield of γ-hydroxyvaline lactone could be expected (see structures X, XI and XII). A further experiment was therefore carried out in which desulphurized cephalosporin C_c was hydrogenated with Adams catalyst before the hydrolysis with HCl.

Cephalosporin C_c (50 mg.) in 4·0 ml. of water was treated with Raney nickel at 20° as described above. Compound 5 was extracted with $CHCl_3$ and the aqueous residue was freed from nickel by treatment with oxine as described previously. A small sample was taken from the solution for the measurement of its extinction at 250 mμ. Adams catalyst (50 mg.) was added to the remainder of solution (3·8 ml.), and the mixture was hydrogenated at atmospheric pressure. After 20 hr. the solution was freed from

Table 1. *Behaviour of degradation products of cephalosporin C and cephalosporin C_c on paper chromatography and electrophoresis*

Electrophoresis was for 1·5 hr. at 14 v/cm. Details of experimental conditions are given in the Methods section. Spots referred to in the Table are described in the Experimental section. Roman numerals refer to structures given in the text.

Product	R_F in system *A*	Mobility* (cm.) at pH 7 towards Anode	Cathode
(IV) (spot 1)	0·054	—	0·7
αβ-Diaminopropionic acid† (spot 2)	0·11	—	6·5
γ-Hydroxyvaline lactone† (XII) (spot 4)	0·46	—	3·4
γ-Hydroxyvaline	0·30	—	0·7
Alanine	0·31	—	0·7
α-Aminoadipic acid	0·33	5·5	—
Valine	0·50	—	0·7
Spot 5	—	—	11·5
Ornithine marker	0·11	—	7·3
Histidine marker	—	—	2·7

* Glucose was carried 0·7 cm. towards the cathode by endosmosis under the conditions used.

† Grey–brown colour with ninhydrin.

the catalyst. The extinction of a sample of this solution at 250 mμ indicated that reduction of the chromophore originally present in desulphurized cephalosporin C_c was about half complete. The solution was then hydrogenated for a further 20 hr. with fresh Adams catalyst (50 mg.). Measurements of ultraviolet-absorption spectra then indicated that the chromophore was virtually destroyed, and that no compound 4 was formed on heating the solution with N-HCl at 100° for 30 min. The solution was evaporated to dryness, the residue was hydrolysed in N-HCl at 110° for 16 hr., and the HCl removed *in vacuo*. The product of hydrolysis was dissolved in 0·5 ml. of water. A sample (8 μl.) of the hydrolysate was subjected to electrophoresis on paper at pH 7·0 and the paper sprayed with ninhydrin. The hydrolysate gave four major spots, due to an acidic substance (α-aminoadipic acid), neutral material and two basic substances. The bases corresponded with spots 4 and 5 (Table 1). A trace of αβ-diaminopropionic acid (spot 2) was also detected.

A small amount of the product responsible for spot 4 was isolated by preparative paper electrophoresis. Fifteen 4 μl. samples of the hydrolysate were spotted on to the paper at 1 cm. intervals. After electrophoresis, three guide strips, spaced equally along the paper, were cut out and sprayed with ninhydrin to locate spot 4. The material in this position was eluted from the remainder of the paper with water. The eluate was evaporated to dryness and the residue dissolved in 24 μl. of water. One half of this solution was heated in a sealed tube with 4 μl. of aq. NH_3 soln. (sp.gr. 0·88) at 100° for 15 min., the contents of the tube were evaporated to dryness, and the residue was dissolved in 12 μl. of water. Samples (4 μl.) of the untreated and NH_3-treated solutions were subjected to electrophoresis on paper at pH 7·0 and paper chromatography in system *A*. The product in the untreated sample was indistinguishable in behaviour from authentic γ-hydroxyvaline lactone (Table 1). The product in the treated sample was indistinguishable from γ-hydroxyvaline.

The basic substance responsible for spot 5 has not been characterized. Conceivably it is 2-hydroxymethyl-*n*-propylamine [$CH_3 \cdot CH(CH_2 \cdot OH) \cdot CH_2 \cdot NH_2$]. The *iso*-butylamide of phenylacetyl-L-alanine is one of the products of hydrogenolysis of benzylpenicillin (Kaczka & Folkers, 1949).

SUMMARY

1. Cephalosporin C ($C_{16}H_{21}O_8N_3S$) has been degraded by hydrolysis, hydrogenolysis and oxidation.

2. Degradation products obtained from one half of the molecule include carbon dioxide, δ-amino-δ-carboxyvalerylglycine, 2-(D-4′-amino-4′-carboxy-*n*-butyl)thiazole-4-carboxylic acid and αβ-diaminopropionic acid.

3. Degradation products obtained from the second half of the molecule include acetic acid, γ-hydroxyvaline, α-oxo*iso*valeric acid and 1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone.

4. Consideration of the degradation products, together with the ultraviolet- and infrared-absorption spectra, nuclear-magnetic-resonance spectrum and behaviour on electrometric titration of cephalosporin C, has led to a definitive structure for the antibiotic. This structure contains a fused β-lactam–dihydrothiazine ring system in place of the β-lactam–thiazolidine ring system of the penicillins.

5. Cephalosporin C_c, formed from cephalosporin C in 0·1 N-hydrochloric acid at room temperature, is deacetylcephalosporin C lactone.

6. The biogenesis of cephalosporin C is considered in relation to that of cephalosporin N and other penicillins.

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We should like to record our thanks to those who informed us, during the course of this work, of their interesting speculations about the structure of cephalosporin C. None of the structures suggested to us contained the dihydrothiazine ring though in one, suggested by Dr W. A. Sexton, the acetoxyl group of cephalosporin C was placed on the γ-carbon atom of a valine-yielding fragment.

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The X-ray Analysis of the Structure of Cephalosporin C

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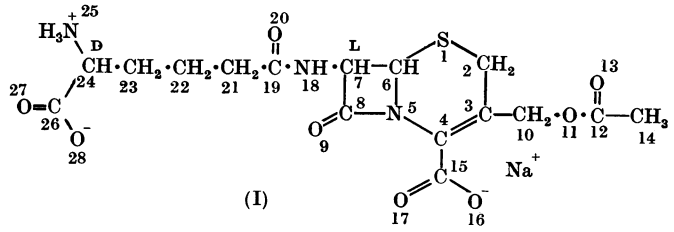
Crystallographic measurements on the sodium salt of cephalosporin C were first made in order to determine the molecular weight of this compound soon after it was isolated by Newton & Abraham (1955). The crystals were then too small for detailed work, but the measurements showed that the lattice had one very short unit-cell dimension of 4·9 Å, a feature that suggested that it might be possible to solve the crystal structure by direct methods, without the introduction of atoms heavier than those already present. As more material was obtained and larger crystals grown, additional unsuspected complications in the crystal lattice were observed and these have hindered the precise solution of the structure. The positions now found for the atoms in the crystal give good general agreement with the observed X-ray intensities but are not defined with great accuracy;



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