Derivatives of Cephalosporin C formed with certain Heterocyclic Tertiary Bases

THE CEPHALOSPORIN C_A FAMILY

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(*Received* 3 *August* 1960)

Cephalosporin C, an acidic antibiotic produced by a *Cephalosporium* sp., has been shown to have the structure (I) (Abraham & Newton, 1961; Hodgkin & Maslen, 1961). When a preparation of cephalosporin C which had been kept overnight in a pyridine–acetate buffer was chromatographed in the same buffer on an anion-exchange resin (Amberlite IR-4B), a substance which showed activity against *Staphylococcus aureus* was present in the first fractions from the column. This substance, named cephalosporin C_A, was well resolved from cephalosporin C itself. When the eluted cephalosporin C was concentrated and rechromatographed later under similar conditions, a further quantity of cephalosporin C_A appeared. It thus seemed that cephalosporin C_A was being formed from cephalosporin C in aqueous pyridine–acetate. Further experiments, which have been briefly reported by Abraham & Newton (1958) and by Hale, Abraham & Newton (1958), showed that the new active substance was the product of a reaction between cephalosporin C and pyridine.

When cephalosporin C was kept in 2M-pyridine–acetate at pH 7 and at temperatures varying from 37° to 70°, the activity of the solution against *Staph. aureus* rose to a value that was several-fold greater than the original one and then declined. Typical changes in activity are shown in Fig. 1. Electrophoresis on paper showed that this rise in activity was associated with the formation of a compound (cephalosporin C_A) that showed no net charge at pH 7·0. The compound was not formed when the pyridine buffer was replaced by the acetates of a number of other bases, including ammonia, trimethylamine, piperidine, or by pyridinium methyl bromide, aniline or *N*-dimethylaniline. It was formed more readily in aqueous pyridine at pH 7 than at pH 5 and its yield increased with the concentration of pyridine to a maximum at about 2M. Further work showed that a family of compounds could be produced by allowing cephalosporin C to react with different heterocyclic tertiary bases. The specific compound obtained with pyridine will be referred to as cephalosporin C_A (pyridine).

On a preparative scale, cephalosporin C_A (pyridine) was separated from remaining cephalosporin C, and from acidic degradation products formed during the reaction, by chromatography on Dowex 1X8 (acetate form). Cephalosporin C_A (pyridine) passed rapidly through the column and was obtained as an amorphous solid on freeze-drying the eluate. This product contained acetate and was extremely hygroscopic. For further purification it was adsorbed on Dowex 50X8 and eluted by stirring the resin with water and adding aqueous ammonia until the pH rose to 7·0. The resulting product was obtained as a non-hydroscopic white powder with an activity of approximately 120 u./mg. against *Staph. aureus* and

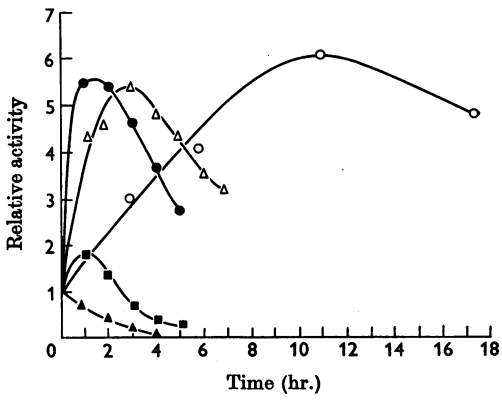

Fig. 1. Changes in activity against *Staph. aureus* with time, of solutions of cephalosporin C sodium salt (10 mg.) in 0·5 ml. of aq. pyridine–acetate (2M to pyridine), pH 7. ○, At 50°; △, at 65°; ●, at 70°; ■, at 80°; ▲, at 90°. Activities are given in arbitrary units.

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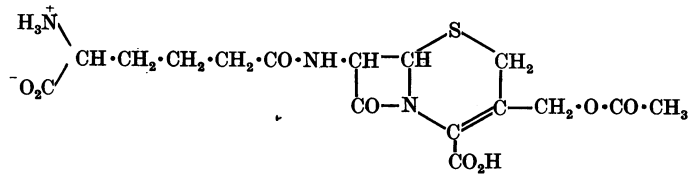
14 u./mg. against *Salmonella typhi* (cephalosporin C sodium salt has an activity of 8–10 u./mg. against both organisms).

Elementary analysis indicated that the product had the composition $C_{19}H_{22}O_6N_4S,3{\cdot}5H_2O$. The product yielded no *C*-methyl in the Kuhn–Roth determination. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 257 mμ ($E^{1\%}_{1\,cm.}$ 244). Its infrared-absorption spectrum showed a strong band at 5·67 μ, but no bands corresponding to those present in cephalosporin C at 5·77, 8·1 and 9·7 μ. Electrometric titration in the pH range 2·5–11·2 revealed an ionizable group with pK_a 9·6 and indicated the presence of at least one group with pK_a < 2·5. When subjected to electrophoresis on paper at pH 5 or 7 the product behaved as though it had no net charge. On paper chromatograms run in butan-1-ol–acetic acid–water its R_F (0·06) was lower than that (0·19) of cephalosporin C. Paper chromatography and electrophoresis indicated that it yielded α-aminoadipic acid on acid hydrolysis. When an acid hydrolysate was made alkaline and distilled, a volatile base was obtained in the distillate whose ultraviolet-absorption spectrum was indistinguishable from that of pyridine.

These properties suggested that cephalosporin C_A (pyridine) had the structure (II) and that its formation involved the replacement of the acetoxyl group in cephalosporin C by a pyridinium ion. Structure (II) was consistent with the N/S ratio of 4/1, the apparent formation of pyridine on hydrolysis, the absence of *C*-methyl, the absence of a band corresponding to an ester carbonyl (5·77 μ) in the infrared spectrum, and with the finding that the product showed no mobility on electrophoresis at pH 7. Alkylation of pyridine, though surprising, appeared feasible, in view of the fact that the acetoxy group formed part of an allylic structure. The pyridinium structure (II) received support from the mobility at pH 7·0 of other members of the cephalosporin C_A family which showed antibacterial activity (Table 1). Most of these compounds were not purified, but their behaviour on paper electrophoresis could be accounted for by the assumption that they were quaternary derivatives of the base used in their production. Thus the compounds formed from quinoline, 2:4:6-trimethylpyridine, 2-hydroxymethylpyridine and nicotinamide showed no net charge at pH 7·0, whereas those formed from pyridinecarboxylic acids migrated towards the anode and those formed from aminopyridines and nicotine migrated towards the cathode. The behaviour of a mixture of three members of the cephalosporin C_A family and of cephalosporin C itself, when subjected to electrophoresis at pH 7·0 followed by paper chromatography in butan-1-ol–acetic acid–water, is shown in Fig. 2.



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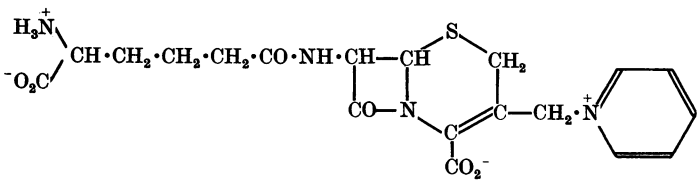

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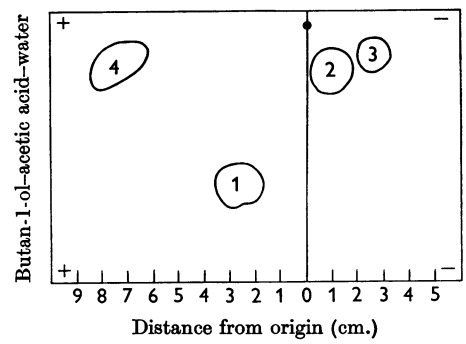


Fig. 2. Separation of cephalosporin C (1) and three members of the cephalosporin C_A family (2–4) by electrophoresis on paper at pH 7 (17 v/cm. for 4 hr.) followed by chromatography in butan-1-ol–acetic acid–water. A sample of the mixture was applied at ●. The cephalosporin C_A compounds were derived from cephalosporin C and pyridine (2), nicotine (3), pyridine-2:3-dicarboxylic acid (4). Spots were revealed by a bioautography with *Salm. typhi* as the test organism.

A compound formed from cephalosporin C and sulphapyridine migrated towards the anode, but less than half as far as cephalosporin C. This is consistent with the acidic nature of the sulphonamido group, which has pK_a 8·3 in sulphapyridine itself (Bell & Roblin, 1942) and would be expected to have a significantly lower pK_a when the pyridine nitrogen acquired a positive charge by conversion into a quaternary derivative. A compound obtained from sulphathiazole and cephalosporin C, whose formation presumably involved the positive charging of the nitrogen atom in the thiazole ring, also migrated towards the anode. The pK_a of the sulphonamido group in sulphathiazole is reported to be 7·12 (Bell & Roblin, 1942).

Further evidence for the quaternary nature of the members of the cephalosporin C_A family was provided by the properties of the compound obtained with nicotinamide. This compound was separated from unchanged cephalosporin C remaining in the reaction mixture by passage through a column of Dowex 1X10 (acetate form) and was obtained as an amorphous powder which showed an activity of 50 u./mg. against *Staph. aureus* and 12 u./mg. against *Salm. typhi*. Its ultraviolet-absorption spectrum in aqueous solution at pH 8 showed $\lambda_{max.}$ at 261 mμ with $E^{1\%}_{1\,cm.}$ 202. On the addition of sodium dithionite the $E_{261\,m\mu}$ fell to 151 and a broad band appeared with $\lambda_{max.}$ at 356mμ and $E^{1\%}_{1\,cm.}$ 96 (Fig. 3). The resulting solution showed a pale green–white fluorescence in ultraviolet light. When the pH of the solution was lowered to 2·0 the band in the ultraviolet-absorption spectrum with $\lambda_{max.}$ 356 mμ disappeared and a new band appeared with $\lambda_{max.}$ 293 mμ with $E^{1\%}_{1\,cm.}$ 218. This type of change in absorption spectrum is characteristic of quaternary nicotinamide derivatives, including the pyridine nucleotides (Warburg & Christian, 1936), the appearance of a band at about 350 mμ on reduction with sodium dithionite being

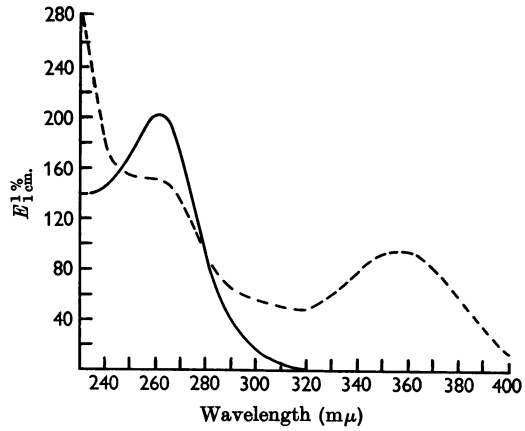


Fig. 3. Changes in the ultraviolet-absorption spectrum of cephalosporin C_A (nicotinamide) on reduction with sodium dithionite in 0·1 M-Na_2HPO_4. —, Before reduction; - - -, after reduction.

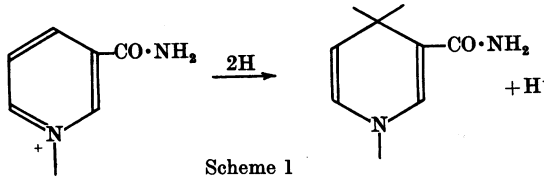
Table 1. *Electrophoretic mobility, R_F values and relative antibacterial activities of cephalosporin C_A derivatives*

Electrophoretic mobility is the distance travelled by the derivative relative to that travelled by cephalosporin C under similar conditions (pH 7·0 in collidine–acetate buffer; 17 v/cm.; 2·5 hr.). +, The derivative migrated towards the anode (negative charge at pH 7); −, migration towards the cathode. Substances with no net charge at pH 7·0 moved towards the cathode approximately 0·4 times the distance that cephalosporin C moved towards the anode. $R_{ceph\ C}$ represents the distances travelled by the derivatives relative to that travelled by cephalosporin C in butan-1-ol–acetic acid–water. Activity ratios represent the quotients [antibacterial activity against *Staph. aureus*/activity against *Salm. typhi*]. The quotient for cephalosporin C is assumed to be 1.

Cephalosporin C_A derived from:	Electrophoretic mobility	$R_{ceph\ C}$	Activity ratio
Nicotine	−1·4	0·16	5·0
2-Aminopyridine	−0·6	0·29	15·0
2-Amino-6-methylpyridine	−0·64	0·6	11·4
Pyridine	−0·4	0·31	10·0
Nicotinamide	−0·4	0·3	4·0
2:4:6-Trimethylpyridine	−0·4	0·54	3·9
2-Hydroxymethylpyridine	−0·4	0·36	3·6
Quinoline	−0·4	0·58	2·7
Sulphapyridine	+0·25	0·55	$\geqslant$1*
Sulphadiazine	+0·25	0·62	$\geqslant$1*
Sulphathiazole	+0·25	0·74	$\geqslant$1*
3-Hydroxypyridine	+0·75	0·36	5·0
*iso*Nicotinic acid	+0·55	0·2	1·30
Nicotinic acid	+1·0	0·23	0·94
Picolinic acid	+0·75	0·36	0·20
Pyridine-2:3-dicarboxylic acid	+3·2	0·33	0·05

* In these cases the inhibition zones on agar seeded with *Salm. typhi* were too small for the activity ratio to be estimated.

due to the reaction in Scheme 1 (Rafter & Colowick, 1954). After chromatography or electrophoresis on paper, cephalosporin C_A (nicotinamide) could be revealed by the procedure described by Kodicek & Reddi (1951) for the detection of quaternary nicotinamide derivatives.



Scheme 1

Different members of the cephalosporin C_A family varied greatly in their relative activities against *Staph. aureus* and *Salm. typhi*. Table 1 shows approximate values for the quotient [activity against *Staph. aureus*/activity against *Salm. typhi*] for a number of compounds, calculated on the assumption that the value for cephalosporin C itself is 1. The quotient was considerably increased when the acetoxy group of cephalosporin C was replaced by a quaternary aromatic base in which the substituents were either non-ionizable groups or groups (such as amino) whose ionization could provide an additional positive charge. This change tended to be reversed when the substituents were negatively charged carboxyl groups, and the nearer the carboxyl to the quaternary nitrogen the greater was its effect. Thus, the compound formed with *iso*nicotinic acid was slightly more active against *Staph. aureus* than against *Salm. typhi*, whereas that formed with picolinic acid was five times as active against *Salm. typhi* as against *Staph. aureus*, and that formed with pyridine-2:3-dicarboxylic acid was about 20 times as active against *Salm. typhi* as against *Staph. aureus*. It may be that these differences in activity are connected with differences in the facility with which the compounds reach their site of action in the two organisms.

EXPERIMENTAL

Antibacterial activities were measured by the hole-plate method (Brownlee *et al.* 1948) with *Staph. aureus* (N.C.T.C. 6571) and *Salm. typhi*, strain 'Mrs S' (Felix & Pitt, 1935) as test organisms and cephalosporin C sodium salt (8–10μ/mg.) as a standard. Analyses were by Weiler and Strauss.

Paper chromatography and electrophoresis. Paper chromatograms were run on Whatman no. 1 paper in (*A*) butan-1-ol–acetic acid–water (4:1:4, by vol.) and (*B*) ethanol–water (7:3, v/v).

Electrophoresis was carried out on Whatman no. 1 paper at 17 v/cm. (for 2·5–4 hr. unless stated otherwise) in aq. collidine–acetate solution (0·05 M to acetate), pH 7·0, pyridine–acetate solution (0·05 M to acetate), pH 5·0, and in 10% (v/v) acetic acid, pH 2·2.

Members of the cephalosporin C_A family were seen as dark spots when the paper was placed before a source of ultraviolet light (230–400 mμ) and appeared as purple spots when the paper was sprayed with ninhydrin. They were also detected by means of bioautographs. The paper was placed on the surface of a plate of nutrient agar seeded with the test organisms. After 15 min. the paper was removed and the plate incubated overnight. Active compounds were located by clear areas where no bacterial growth had occurred. The compounds formed from cephalosporin C and sulphapyridine or sulphathiazole could also be detected by spraying the paper with 0·2% of $NaNO_2$ in 0·1N-HCl, drying at 60° and then spraying with 1% (w/v) α-naphthylamine in 75% (v/v) acetic acid. Aromatic amines give red spots under these conditions (Eckman, 1948). The compound formed from cephalosporin C and nicotinamide could be detected by suspending the paper for 1 hr. in the vapour of butan-2-one–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v). It then appeared as a blue–white fluorescent spot when the paper was viewed in ultraviolet light (365 mμ) (Kodicek & Reddi, 1951).

Small-scale production of members of the cephalosporin C_A family. Cephalosporin C sodium salt (10 mg.) was dissolved in an aqueous solution (0·50 ml.) of the base used. The concentration of the base was between 0·1 and 2·0 M and the pH of the solution was between 4·5 and 7·0, being adjusted (if necessary) to a value within this range by the addition of acetic acid. With sulphapyridine and sulphathiazole, whose solubility in water is low, the reaction was carried out in 50% (v/v) dimethylformamide. The mixture was kept at 37° and after different intervals (usually 24 and 48 hr.) 5μl. samples were spotted on paper for analysis by electrophoresis or chromatography or both. Bioautographs revealed the cephalosporin C_A and remaining cephalosporin C. Spraying with ninhydrin revealed cephalosporin C_A, cephalosporin C and usually also some 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (Jeffery, Abraham & Newton, 1960).

Estimations of relative antibacterial activities after paper chromatography or electrophoresis. Semi-quantitative estimates of the relative activities of members of the cephalosporin C_A family against *Staph. aureus* and *Salm. typhi* were obtained by the application of techniques similar to those used for the penicillins by Goodall & Levi (1947). A solution (10μl.) containing a mixture of cephalosporin C and a cephalosporin C_A compound was spotted on a strip of Whatman no. 1 paper in sixfold replication. The components were separated by chromatography or electrophoresis, and, after the solvent or buffer had been evaporated in a stream of air, the paper strip was applied to nutrient-agar sheets (30 cm. × 50 cm.) seeded with one of the test organisms. At the same time paper strips bearing standard spots of cephalosporin C in three graded amounts, each in sixfold replication, were applied to the sheet in such a way as to distribute the resulting inhibition zones at random about the agar sheet. After 15 min. the papers were removed and the agar sheets incubated overnight. The resulting inhibition zones were approximately elliptical and both axes were measured and their geometric means calculated. The mean diameters of the standard spots were plotted against the logarithm of the concentration. The relative activity of test spots was determined by applying their mean axes to the graph so formed. The accuracy of such a method was not high and assumed a

parallelism between log concentration/zone diameter for both cephalosporin C and the members of the C_A family which may not exist. However, it gave an indication of the relative activities of the derivatives against the two organisms, compared with the activity of cephalosporin C, before the derivatives had been isolated in quantity from reaction mixtures.

Preparation and purification of cephalosporin C_A (pyridine). A solution of cephalosporin C sodium salt (1 g.) in water (50 ml.) was brought to pH 2·5 by the addition of Dowex 50X8 (H form). About 1 g. of damp resin was required. The resin was removed by filtration and washed with a little water. Pyridine (8 ml.) was added to the combined filtrate and washing. The solution was kept at 46° for 18 hr. and then freeze-dried. The residue was dissolved in the minimum amount of water (about 2 ml.) and precipitated by the addition of acetone (about 50 ml.). The precipitate was centrifuged down and ground under dry acetone to form a pale buff-coloured powder. The powdered material was dissolved in water (about 2 ml.) and added to a column (9 cm. × 2 cm. diam.) of Dowex 1X10 (200–400 mesh; acetate form). Water was then allowed to flow through the column and the cephalosporin C_A (pyridine) emerged in the first 30 ml. of eluate. The latter was freeze-dried and the residue stirred with dry acetone. The resulting powder, freed from acetone *in vacuo*, weighed 250 mg. It showed an activity of 80 u./mg. against *Staph. aureus* and its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 257 mμ with $E^{1\%}_{1\,cm.}$ 180. The very hygroscopic product could be kept in a desiccator over P_2O_5.

A sample of this product (200 mg.) was dissolved in water (2 ml.) and the solution added to a column (5 cm. × 1 cm. diam.) of Dowex 50X8 (200–400 mesh; H form). The solution was applied to the column under slight air pressure and the column then washed rapidly with water (8 ml.). The resin was then removed from the column and stirred with water (about 25 ml.) while aq. NH_3 soln. was added [aq. NH_3 soln. (sp.gr. 0·88) diluted 1 to 4] until the pH rose to 6·9. The resin was filtered off and the clear filtrate freeze-dried. The residue was stirred with acetone, collected by centrifuging and dried *in vacuo* (118 mg.). This product was an almost white non-hygroscopic powder with an activity of 110–120 u./mg. against *Staph. aureus*. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 257 mμ with $E^{1\%}_{1\,cm.}$ 244 (Found: C, 46·0; H, 6·2; N, 11·2; S, 5·8. $C_{19}H_{22}O_6N_4S,3{\cdot}5H_2O$ requires C, 46·0; H, 5·9; N, 11·2; S, 6·4%). When subjected to electrophoresis on paper at pH 5 or 7 it behaved as though it had no net charge; at pH 2·2 it migrated towards the cathode.

Preparation of cephalosporin C_A (nicotinamide). Cephalosporin C sodium salt (100 mg.) was dissolved in 5 ml. of water and Dowex 50X8 (H form) was added to the stirred solution until the pH fell to 2·6. (About 70 mg. of resin was required.) The resin was removed by filtration and washed with a little water. Nicotinamide (610 mg.) was dissolved in the combined filtrate and washing, when the pH rose to about 4·8. The solution was kept at 37° for 48 hr. and then freeze-dried. The residue was stirred three times with 10 ml. of acetone (to extract remaining nicotinamide) and the insoluble material separated each time by centrifuging. The dried material (74 mg.) was dissolved in about 1 ml. of water and added to a column (10 cm. × 1 cm. diam.) of Dowex 1X10 (200–400 mesh; acetate form). Water was allowed to pass through the column and 1 ml. fractions were collected. Fractions 3–6, which contained ninhydrin-positive material, were combined and freeze-dried. The product was stirred with 2 ml. of acetone, separated from the acetone by centrifuging, and dried *in vacuo* (28 mg.). It showed an activity of 50 u./mg. against *Staph. aureus* and 12 u./mg. against *Salm. typhi*. It gave a single spot (detected by bioautograph, ninhydrin, fluorescence with butan-2-one–NH_3 or absorption of ultraviolet light) when chromatographed on paper in system *A* (R_F 0·04), or in system *B* (R_F 0·43) and a single spot corresponding to a substance with no net charge when subjected to electrophoresis on paper at pH 7·0. It migrated 3 cm. towards the cathode on electrophoresis at pH 2·2.

Reduction with sodium dithionite. Cephalosporin C_A (nicotinamide) (1·15 mg.) was dissolved in 3·5 ml. of 0·1 M-Na_2HPO_4 (pH 8). A sample (0·5 ml.) of the solution was diluted to 3 ml. with 0·1 M-Na_2HPO_4 and the ultraviolet-absorption spectrum of the diluted solution was measured. The remainder of the original solution was bubbled through with N_2, 3 mg. of $Na_2S_2O_4$ was added, and the solution left in a closed tube for 50 min. The solution was then diluted to 9 ml. with 0·1 M-Na_2HPO_4, and O_2 was passed through it for 5 min. A sample was then diluted with an equal vol. of 0·1 M-Na_2HPO_4 and its ultraviolet-absorption spectrum measured (Fig. 3).

SUMMARY

1. Cephalosporin C reacts with pyridine in neutral aqueous solution to form a new compound with antibacterial activity. This compound shows no net charge on paper electrophoresis at pH 5–7. It has been named cephalosporin C_A (pyridine).

2. Cephalosporin C reacts in a similar fashion with a number of derivatives of pyridine, including nicotinamide, pyridinecarboxylic acids and sulphapyridine, and also with sulphathiazole and sulphadiazine. The resulting family of compounds has been named the cephalosporin C_A family.

3. Evidence is presented that the formation of members of the cephalosporin C_A family involves the displacement of the acetoxy group in cephalosporin by the heterocyclic tertiary base and conversion of the latter into a quaternary derivative.

4. The relative activity of members of the cephalosporin C_A family against *Staphylococcus aureus* and *Salmonella typhi* varies with the base from which they are formed. The compound formed with pyridine is at least 12 times as active as cephalosporin C against *Staphylococcus aureus* but less than twice as active against *Salmonella typhi*. The compound formed with pyridine-2:3-dicarboxylic acid is more than 20 times as active against *Salmonella typhi* as against *Staphylococcus aureus*.

We are indebted to Mrs M. Loveridge, Mr O. Boys, and Mr D. Gazzard for skilful technical assistance.

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The Cephalosporin C Nucleus (7-Aminocephalosporanic Acid) and some of its Derivatives

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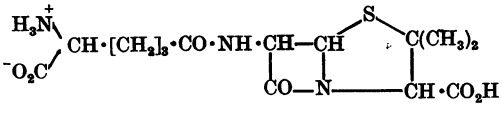
(*Received* 3 *August* 1960)

Cephalosporin N (I) (Newton & Abraham, 1954) may be regarded as a derivative of 6-aminopenicillanic acid (II) (Sheehan, Henery-Logan & Johnson, 1953) in which the 6-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid. Cephalosporin N has only about 1% of the antibacterial activity of benzylpenicillin against *Staphylococcus aureus* (Abraham, Newton & Hale, 1954).

Cephalosporin C (III) may be regarded as a derivative of 7-aminocephalosporanic acid (IV) in which the 7-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid (Abraham & Newton, 1961). Thus cephalosporin C and cephalosporin N have the same side chain, but different nuclei (II and IV respectively). Cephalosporin C has only about 10% of the activity of cephalosporin N against *Staph. aureus* (Newton & Abraham, 1956).

In view of these relationships it seemed probable that an analogue of cephalosporin C, which had a phenylacetyl group instead of a D-(δ-aminoadipoyl) group as a side chain, would be about 100 times as active as cephalosporin C and about one-tenth as active as benzylpenicillin against *Staph. aureus*. The preparation and *N*-acylation of 7-aminocephalosporanic acid provided one possible route to such an analogue.

In the course of work on the chemical structure of cephalosporin C, it became clear that this compound did not undergo a penicillin–penillic acid type of rearrangement and that its β-lactam ring was considerably more stable, under acid conditions, than the β-lactam ring of the penicillins.



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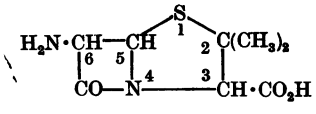

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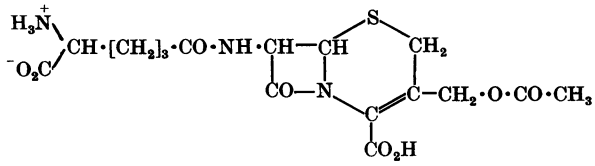
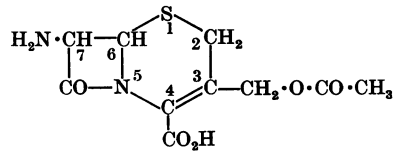


(III)



(IV)