THE CEPHALOSPORINS

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I. INTRODUCTION

Among the widespread searches for chemotherapeutic antibiotics which followed the demonstration of the clinical value of penicillin (47) was one by Professor G. Brotzu of Cagliari, Sardinia. In 1945 Brotzu isolated from the sea near a sewage outfall off the Sardinian coast an antibiotic-producing organism which he identified as a species of *Cephalosporium* similar to *Cephalosporium*

acremonium. When this organism was subcultured on nutrient agar, or on a stationary liquid medium, it secreted material which inhibited the growth of a number of gram-positive and gram-negative bacteria, including *Staph. aureus* and *Salm. typhi*. Brotzu reported that culture fluids containing the active material exerted a beneficial effect when used locally to treat staphylococcal infections in man, or given systemically to patients with typhoid fever.

In an account of his work, which appeared only in a local and little known journal (32), Brotzu remarked that the antibiotic which he had encountered was likely to prove difficult to purify and he expressed the hope that it would be studied in laboratories with better facilities than were available in Sardinia. In 1948 Brotzu's experiments were brought to the attention of Sir Howard Florey by a former British Public Health Officer in Sardinia and, in consequence, work on the *Cephalosporium* sp. and its products was undertaken first at Oxford and subsequently at the Medical Research Council's Antibiotics Research Station at Clevedon, Somerset.

Although Waksman and Horning had stated in 1943 that some members of the *Fusarium-Cephalosporium* group were decidedly antagonistic to the growth of bacteria on a solid medium (107), Brotzu appears to have made the first specific study of an antibacterial substance from a species of *Cephalosporium*. However, in 1951 the production of an antibiotic by a member of the genus *Tilachlidium* and by *Cephalosporium charticola* was reported in the U. S. A. (50). It subsequently appeared that the *Tilachlidium* was a new species of *Cephalosporium*. This species was named *Cephalosporium salmosynnematum* (92) and the antibiotic it produced was named first synnematin (50) and later synnematin B (82).

In the first experiments at Oxford, N. G. Heatley found that an acidic antibiotic which was readily extractable into organic solvents was present in culture fluids of the Sardinian *Cephalosporium* sp. (Commonwealth Mycological Institute, Kew [C.M.I.] No. 49, 137). This substance was isolated by Burton and Abraham (33) from culture fluids produced at the Antibiotics Research Station, Clevedon, where it was named cephalosporin P because it was active mainly against gram-positive bacteria. Structural studies on cephalosporin P showed later that it probably belonged to the steroid group.

Some of the properties of cephalosporin P appeared to differ from those of the active material described by Brotzu. It was therefore not surprising when a second and entirely different antibiotic was discovered in culture fluids from which cephalosporin P had been extracted (33, 39). The new substance, which was an acid and extremely hydrophilic, was named cephalosporin N because it showed activity against gram-negative, as well as gram-positive, bacteria. Its isolation proved to be troublesome but it was eventually obtained by Abraham, Newton and Hale (4) in a nearly pure form. Its properties indicated that it was a new type of penicillin with a side-chain derived from D-α-aminoadipic acid (3, 75, 76). Synnematin B was subsequently shown to be identical with cephalosporin N (12).

Partially purified preparations of cephalosporin N were found by Newton and

Abraham (78) to be contaminated with a chemically related substance, named cephalosporin C, which could be readily isolated as a crystalline sodium salt. Cephalosporin C resembled cephalosporin N in its antibacterial range, but against most organisms it was considerably less active than the latter. Because of its low activity and because it was produced in only very small amount by *Cephalosporium* sp. C.M.I. 49,137, cephalosporin C could not be detected, at the time of its discovery, in the culture fluids from which it was concentrated during the purification of cephalosporin N. It would thus have escaped separate notice in a conventional screening programme for antibiotic activity.

In 1957 Grosklags and Swift reported that they had observed the perfect stage of *Cephalosporium salmosynnematum* and they classified this organism as a new species of the genus *Emericellopsis* van Beyma (*E. salmosynnematum*) (51). A number of species of *Emericellopsis* were then shown to produce cephalosporin N (synnematin B) (51, 67). Only cephalosporin P was found in culture fluids of *E. humicola*. Cephalosporin C was not reported to be formed by any of the species of *Emericellopsis* that were studied (67). Cephalosporin N is also formed by a member of the genus *Streptomyces* (71a).

Since cephalosporin P is unrelated in structure to cephalosporin N and cephalosporin C it will be considered separately here. Cephalosporin N, as a true penicillin, may appropriately be named penicillin N (45).

II. CEPHALOSPORIN P

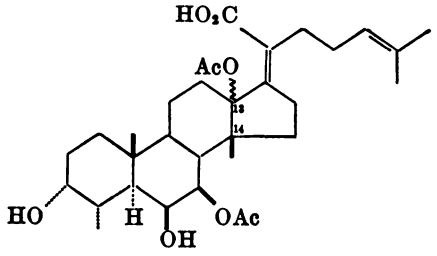
A. Production and isolation

Cephalosporin P was produced in deep and strongly aerated cultures of *Cephalosporium* sp. C.M.I. 49,137 in a medium containing corn steep liquor and glucose (39). The culture fluid was extracted with butyl acetate, at pH 6.5, and the active material in the extract was purified by countercurrent distribution in a system composed of hexane, di*iso*propyl ether, acetone, and phosphate buffer, pH 6.0, followed by chromatography on Florisil in solvents of increasing polarity (33). During this work it was found that the active material consisted of one major component (P_1) and at least four minor components (P_2 to P_5). Cephalosporins P_1, P_2, and P_4 were isolated in crystalline form, but only P_1 was obtained in sufficient quantity for detailed investigation.

B. Chemical structure

Burton, Abraham and Cardwell (34) showed that cephalosporin P_1 ($C_{32}H_{48}O_8$) was a monocarboxylic acid containing two acetoxyl groups, two hydroxyl groups, one easily reducible double bond, and one double bond which was difficult to reduce. The ultraviolet absorption spectra of the antibiotic and its dihydro derivative indicated that the second double bond was part of an α,β-unsaturated carboxylic acid grouping. After hydrolysis of the antibiotic with alkali, acidification yielded a dideacetyl lactone. These findings indicated that cephalosporin P_1 was a tetracyclic compound with a C_{28} skeleton and suggested that it might be a steroid.

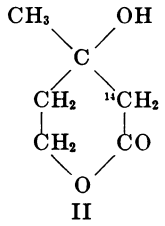
Further work by Baird *et al.* (18) showed that ozonolysis of cephalosporin P_1 methyl ester yielded acetone and that ozonolysis of dihydrocephalosporin P_1 methyl ester yielded a ketone which afforded an α,β-unsaturated ketone on treatment with acid. Monodeacetylcephalosporin P_1 was shown to contain an α-glycol grouping. The results of these and other studies, in which use was made of nuclear magnetic resonance spectra and optical rotatory dispersion to throw light on the nature of degradation products, were consistent with the structure I for cephalosporin P_1.



I

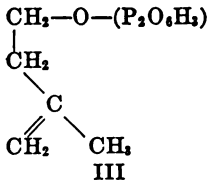
C. Biosynthetic relationships

When [2-^{14}C] mevalonic lactone (II) was added to the culture fluid of the *Cephalosporium* sp., it was incorporated into the cephalosporin P_1 formed (18).

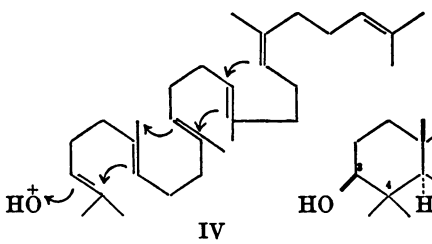


II

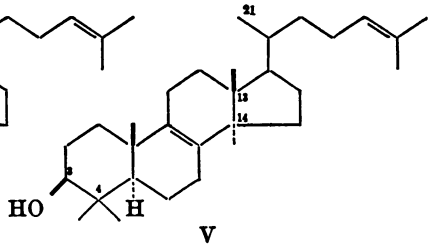
This was consistent with the proposed steroid structure for the antibiotic. Mevalonic acid is known to be a precursor of members of the sterol-triterpene family in both animal cells and micro-organisms (106). In the formation of lanosterol, mevalonic acid is converted, *via* *iso*pentenyl pyrophosphate (III), to squalene (IV) and the latter undergoes a concerted shift of electrons, accompanied by the 1,2-migration of two methyl groups, to yield a tetracyclic structure (V). Lanosterol is converted to cholesterol by processes which include the removal



III



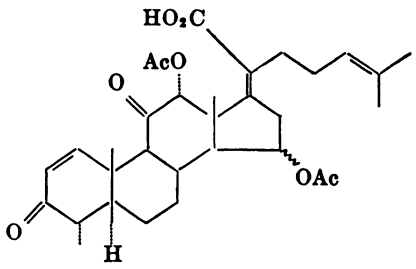
IV



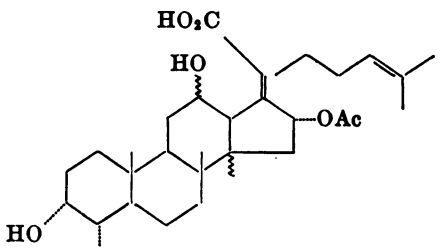
V

of methyl groups at C-4, 4 and 14. However, structure I contains a 14β instead of a 14α methyl group and in this its skeleton resembles tirucallane rather than lanostane. Structure I is also unusual in containing an acetoxy group, rather than a methyl group, at C-13. Special problems therefore arise in connection with its biosynthesis.

Several acidic steroids are produced by fungi, including eburicoic acid which has a carboxyl group at C_{21} (58). But only one fungal steroid with strong antibacterial activity appears to have been isolated before cephalosporin P_1. This is helvolic acid ($C_{32}H_{42}O_8$), which was obtained by Chain, Florey, Jennings and Williams in 1943 from the culture fluid of *Aspergillus fumigatus* mut. *helvola* Yuill (35, 109). Burton, Abraham and Cardwell (34) showed later that helvolic acid was a tetracyclic compound which contained two acetoxy groups and an α,β-unsaturated carboxyl group, but that it had one more double bond than cephalosporin P_1 and two keto groups in place of the two hydroxyl groups of the latter. In 1956 Cram and Allinger tentatively proposed a structure for helvolic acid which was unusual in that it contained no oxygen function at C-3 (38). Later Allinger and Coke suggested the new structure VIa (14). The side-chain of this structure is similar to that of the structure proposed for cephalosporin P_1 (I), but the two structures differ considerably in other respects.



VIa



VIb

More recently, a strongly antibacterial acidic steroid named fucidin has been shown to be produced by *Fusidium coccineum* (K. Tubaki) (50a). Fucidin has been tentatively assigned the structure VIb. It thus appears to have the same side-chain as cephalosporin P_1 and helvolic acid. It differs from cephalosporin P_1, however, in containing only one acetoxy group and in the distribution of other substituents on the steroid ring system.

D. Antibacterial properties

Low concentrations of cephalosporin P_1 inhibited the growth *in vitro* of staphylococci, corynebacteria, and *Cl. tetani*. For example, in tests with a small inoculum the antibiotic was inhibitory to a number of strains of *Staph. aureus* at 1 μg/ml or less. On the other hand, it had little effect on streptococci, gram-negative bacteria, or *Myco. tuberculosis*. In general, the antibacterial range of cephalosporin P_1 is similar to that of helvolic acid and fucidin. But, while cephalosporin P_1 is about twice as active as helvolic acid against a number of organisms (91), it is only about one-tenth as active as fucidin (19a, 55a). Combinations of

cephalosporin P_1 and benzylpenicillin or of fucidin and benzylpenicillin act synergistically against many strains of penicillinase-producing staphylococci (19a).

Staphylococci rapidly acquired resistance to cephalosporin P_1, as they did to helvolic acid, when subcultured in the presence of the drug. Organisms that had acquired resistance to cephalosporin P_1 showed cross resistance to helvolic acid and *vice versa* (33). The facility with which resistant organisms emerged from a large population of staphylococci may have been responsible for the fact that both substances were considerably more effective in suppressing the growth of a small inoculum than that of a large one. Resistant staphylococci also emerge readily in the presence of fucidin and these organisms show cross resistance to cephalosporin P_1 (19a).

Dihydrocephalosporin P_1, in which the double bond in the side-chain of structure I had been saturated, was as active against *Staph. aureus* as was cephalosporin P_1 itself. On the other hand, monodesacetylcephalosporin P_1 showed only about 12% of the activity of the parent compound (34). Both cephalosporin P_1 and helvolic acid were inactivated by an enzyme, or enzymes, present in a crude preparation of penicillinase and in culture filtrates of several strains of aerobic spore-forming bacilli (33). It seems possible that the loss of activity was due to the removal of one or both of the O-acetyl groups in the molecule.

E. Pharmacological and therapeutic properties

When given intravenously, cephalosporin P_1 was less toxic than aureomycin, the LD50 for mice being between 500 and 750 mg/kg. It was well absorbed from the gastrointestinal tract and after oral administration serum levels were readily obtained which were higher than those required to inhibit the growth *in vitro* of *Staph. aureus*. Nevertheless, the drug was considerably less effective than aureomycin or terramycin in protecting mice from staphylococcal infections. It increased the length of life, but not the number of animals which ultimately survived unless the infections were produced with small inocula (45, 91). In this respect, the results of the protective experiments resembled those obtained earlier with helvolic acid (47).

The reason for the relatively poor performance of cephalosporin P_1 in animals was not ascertained. It did not appear to be due to the development of resistant organisms, since staphylococci recovered from treated mice were as sensitive to the drug as the organisms used for infection. Possibly cephalosporin P_1 is handicapped by the fact that it is predominantly bacteriostatic rather than bactericidal in low concentrations (2). Possibly it is inactivated in animals by the action of an acetyl esterase, for although it disappeared from the blood rather quickly, little appeared in the urine or bile. The question arises whether analogues of cephalosporin P_1, in which, for example, the acetoxy groups have been replaced by other substituents, would be more effective chemotherapeutic agents than the parent antibiotic. It is of interest in this connection that fucidin, which has a higher activity *in vitro* than cephalosporin P_1, has been used successfully in the treat-

ment of infections caused by penicillin-resistant staphylococci in man (50a, 96a, 102a).

III. CEPHALOSPORIN N (PENICILLIN N) AND CEPHALOSPORIN C

A. Production and purification

1. Production of penicillin N. Penicillin N was produced by *Cephalosporium* C.M.I. 49,137 in deep culture with high rates of aeration in a medium containing sucrose, corn steep liquor, and ammonium acetate (48). With *E. salmosynnematum* a medium was used which contained maize and soya bean meal, ammonium sulphate, and calcium carbonate (86). Chemically defined media were also used (26, 53). The yield of penicillin N was increased by the addition to the medium of betaine or choline chloride (53) and particularly of methionine (66, 71). D-Methionine was more effective than the L-isomer with both *Cephalosporium* C.M.I. 49,137 and *E. salmosynnematum*. However, two species of *Emericellopsis* which produced penicillin N were reported not to respond to methionine (67).

2. Production of cephalosporin C. The yield of cephalosporin C in fermentations with *Cephalosporium* C.M.I. 49,137 was much smaller than that of penicillin N. A series of mutants of this organism, produced by irradiation with ultraviolet light, was therefore screened at the Antibiotics Research Station, Clevedon, and a strain was eventually isolated (mutant 8650) which yielded many times as much cephalosporin C (but not penicillin N) as did the parent in the same medium. With the isolation of this mutant the production of cephalosporin C in quantity first became a practicable undertaking.

3. Isolation of penicillin N. The methods that could be used for the purification of penicillin N were restricted by the lability of the substance at pH values below 5 or above 9 and by its insolubility in most organic solvents. One process involved adsorption of the antibiotic from the culture fluid onto a column of activated charcoal equilibrated with phosphate buffer, pH 6, and elution with 80% aqueous acetone, followed by chromatography on acid-washed alumina in 80% aqueous acetone and elution with 20% aqueous acetone or dilute alkali. Subsequent countercurrent distribution in a carbon tetrachloride–phenol–water system, pH 6, containing collidine sulphate as a buffer and carrier, led to a barium salt which appeared to be about 80% pure (4). Other methods involved adsorption onto and elution from charcoal (84) and chromatography on ion-exchange resins (37). Fusari and Machamer (49) used chromatography in 70% *iso*propanol on cellulose powder and obtained a preparation of penicillin N that was almost pure. The N-acetyl derivative of this product yielded a crystalline N,N-dibenzylethylenediamine salt ($C_{32}H_{43}O_7N_5S$).

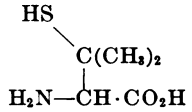
4. Isolation of cephalosporin C. Cephalosporin C was isolated more easily than penicillin N because it was much more stable than the latter in dilute acid solution and readily formed a crystalline sodium salt. It was first encountered during chromatography on Amberlite IR4B of the crude penillic acid obtained by keeping a solution of partially purified penicillin N at pH 2.7. With ammonium acetate or ammonium formate buffer as eluents, cephalosporin

C emerged from the column, after the penillic acid, as a band of ninhydrin-positive material the ultraviolet absorption spectrum of which showed λmax. at 260 mμ (79). Subsequently it was isolated directly from culture fluids of *Cephalosporium* sp. C.M.I. 49,137 (mutant 8650). Penicillin N formed during the fermentation was converted to its penillic acid by incubation of the culture fluid at pH 3. Cephalosporin C was separated from the penillic acid and other products by chromatography in pyridine acetate or ammonium acetate buffer on anion exchange resins and was obtained in crystalline form as a sodium salt (105).

Cephalosporin C was separated less easily from penicillin N than from the isomeric penillic acid, but the two antibiotics were partly resolved by chromatography on Amberlite IR4B and completely resolved by chromatography on paper (79). Cephalosporin C can be located on paper chromatograms by bioassay, by coloration with ninhydrin, by its absorption of ultraviolet light (79), and by a procedure which depends on the fact that it is inactivated by alkali with the formation of a product which absorbs about four equivalents of iodine (13, 99, 103).

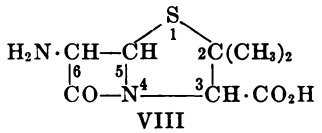
B. Chemical structure and properties

1. Structure of penicillin N. Soon after it had been distinguished from cephalosporin P, penicillin N was found to be inactivated by a crude preparation of the enzyme penicillinase. At the time, however, the significance of this finding was uncertain. The enzyme preparation that was used also inactivated cephalosporin P and therefore contained at least one enzyme in addition to penicillinase. Moreover, no penicillin previously encountered had such strongly hydrophilic properties as the product of the *Cephalosporium*. The first convincing evidence that the latter was indeed a penicillin came from the identification of the amino acid penicillamine as a product of hydrolysis of partly purified material (3, 75). Penicillamine (D-β-thiolvaline, VII) is a characteristic degradation product of the penicillins.



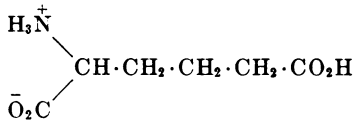
VII

Penicillin N contained a free amino group and behaved on electrometric titration as a monoaminodicarboxylic acid (77). Elementary analyses of a highly purified barium salt of the antibiotic (77) and of the crystalline dibenzylethylenediamine salt of its N-acetyl derivative (49) were consistent with a molecular formula $C_{14}H_{21}O_6N_3S$. On the assumption that penicillin N was an N-acyl derivative of the characteristic penicillin nucleus, which has been named 6-aminopenicillanic acid (6-APA) and has the structure VIII, six carbon atoms and one



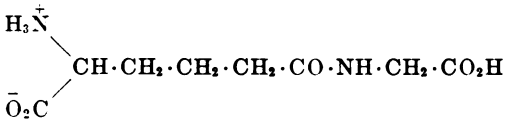
VIII

nitrogen atom remained to be accounted for in the side-chain. This picture was consistent with the finding that the antibiotic yielded D-α-aminoadipic acid



IX

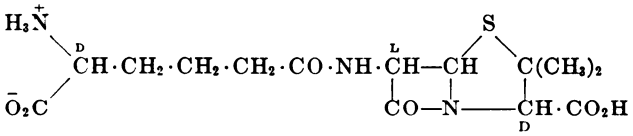
(IX), in addition to penicillamine and CO_2, on acid hydrolysis. The α-aminoadipic acid appeared to be linked to the rest of the molecule through its δ-carboxyl group, since penicillin N showed a positive ninhydrin reaction and an ionizable group the pKa of which was 9.8. This pKa value was close to that for the amino group of free α-aminoadipic acid, and considerably higher than would have been expected if the α-carboxy group of the α-aminoadipic acid residue had been involved in peptide linkage.



X

The way in which the side-chain was joined to the nucleus was finally established by the characterization of a product that was formed when penicillin N was subjected to acid hydrolysis and the neutral fraction of the hydrolysate was oxidized with silver oxide. This product was shown to be D-δ-(α-aminoadipoyl)-glycine (X) and its structure was confirmed by synthesis (5). Its formation was accounted for by hydrolysis of the penicillin ring system to yield penicillamine and a β-aldehydo acid, loss of CO_2 from the latter, and oxidation of the aldehyde group to a carboxylic acid.

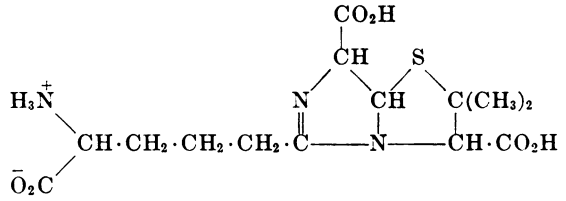
These and other properties of penicillin N have left no doubt that this substance is D-(4-amino-4-carboxybutyl)penicillin (XI) (8, 77). Like other penicillins



XI

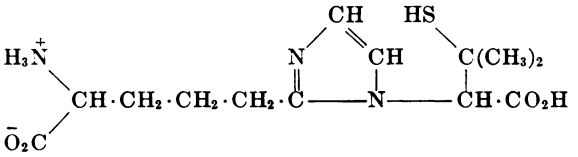
it is an N-acyl derivative of 6-aminopenicillanic acid (VIII) (20, 97). Unlike the common penicillins, however, it contains a zwitterionic side-chain which gives it extremely hydrophilic properties. The amino group of this side-chain can readily be acylated under conditions which do not affect the remainder of the molecule (77).

In aqueous solution at pH 3, penicillin N isomerized to the corresponding



XII

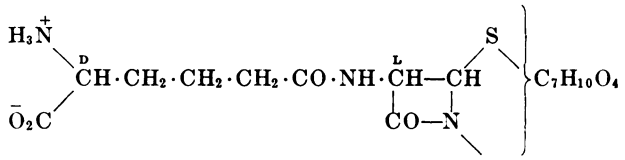
penillic acid (XII), the ultraviolet absorption spectrum of which showed λmax. at 240 mμ. On treatment with mercuric chloride the penillic acid was converted to a penillamine (XIII) which was isolated as a crystalline S-benzyl derivative (77). In these reactions penicillin N resembled benzylpenicillin (6-phenacetamidopenicillanic acid).



XIII

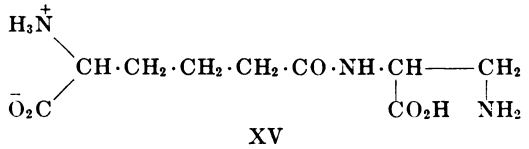
2. Structure of cephalosporin C. Cephalosporin C ($C_{16}H_{21}O_8N_3S$) contained two carbon atoms and two oxygen atoms more than penicillin N(6). Like penicillin N, cephalosporin C behaved as an amino dicarboxylic acid and showed an infrared absorption spectrum with a strong band at 5.61 μ. A band in this position is characteristic of the infrared absorption spectra of the penicillins and is associated with the stretching vibration of the C=O moiety of the fused β-lactam ring.

A number of degradation products of cephalosporin C indicated that it contained the partial structure XIV. Thus, it yielded D-α-aminoadipic acid and



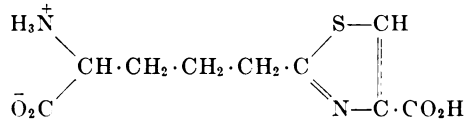
XIV

CO_2 on hydrolysis with hot acid, and D-α-aminoadipic acid and L-alanine, among other products, when the hydrolysis was preceded by hydrogenolysis with Raney nickel. On treatment with Raney nickel and partial hydrolysis of the product with acid, it yielded a dipeptide of D-α-aminoadipic acid and α,β-diaminopro-



XV

pionic acid (XV) (64). When kept in neutral aqueous solution at 37° cephalosporin C was partly converted to D-2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (XVI), the structure of which was confirmed by synthesis (64). The formation of this thiazole from the partial structure (XIV) could be accounted for by opening of the β-lactam ring, fission of the bond between sulphur and the remainder of the molecule, and a nucleophilic attack of the sulphur on the amide carbon of the side-chain.



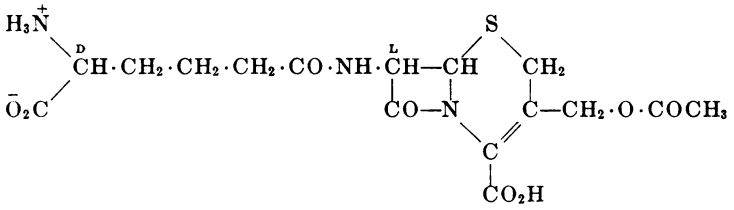
XVI

Two of the seven unplaced carbon atoms of the partial structure XIV were present in an acetoxyl group. The remaining five atoms formed the carbon skeleton of valine, since DL-valine and some α-keto*iso*valeric acid were obtained, in addition to D-α-aminoadipic acid and L-alanine, when the antibiotic was subjected to hydrogenolysis with Raney nickel and the product hydrolysed with hot acid. On similar treatment, penicillin N yielded D-valine by removal of sulphur from the penicillamine fragment of the molecule.

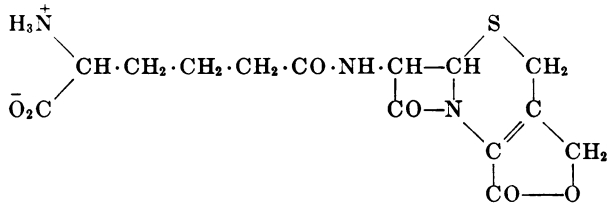
The way in which a valine-yielding fragment was incorporated into the structure of cephalosporin C proved somewhat difficult to elucidate. Cephalosporin C differed strikingly from penicillin N in that it produced no penicillamine on hydrolysis but yielded sulphur-containing products which were non-nitrogenous. A first hypothesis was that a residue of α-hydroxypenicillamine (or its O-acetyl derivative) was present in the molecule (6). However, this hypothesis made no provision for the presence of a chromophore which was necessary to account for the strong absorption of cephalosporin C at 260 mμ (ϵ = 9,600) and in due course it was abandoned.

An important step forward was made when a nuclear magnetic resonance spectrum of cephalosporin C, determined by Dr. R. E. Richards and Mr. T. Higham, showed that no gem-dimethyl group ($C(CH_3)_2$) was present in the molecule. The presence of a single C—CH_3 group, which had been revealed by a Kuhn-Roth determination, was accounted for by the acetoxyl group. At this stage the structure XVII was suggested by Abraham and Newton for cephalosporin C as a working hypothesis (10, 11). Structure XVII contained a fused dihydrothiazine-β-lactam ring system in place of the thiazolidine-β-lactam ring system of the penicillins. It accounted for the fact that cephalosporin C yielded no penicillamine on hydrolysis but yielded DL-valine and α-keto*iso*valeric acid when hydrolysis was preceded by hydrogenolysis with Raney nickel. It contained a conjugated double bond which would result in an absorption band in ultraviolet light; although such a chromophore might not have been predicted to show λmax. at as long a wavelength as 260 mμ, no ring system was known on which a close analogy could be based. Structure XVII also accounted for the formation of a neutral compound, known as cephalosporin Cc, when cephalo-

sporin C was kept in N HCl at room temperature. This compound, which showed no net charge when subjected to electrophoresis at pH 7, could be formulated as the lactone of deacetylcephalosporin C (XVIII). Deacetylcephalosporin C itself was obtained by the action of an acetyl esterase from orange peel on cephalosporin C. The deacetyl derivative lactonized readily in N HCl (65).

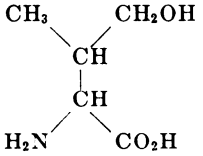


XVII

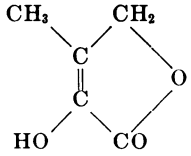


XVIII

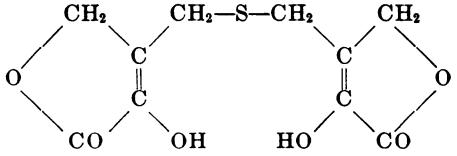
Soon after structure XVII was first suggested, Professor Dorothy Hodgkin and Dr. T. M. Maslen, who had been undertaking an X-ray crystallographic study of cephalosporin C sodium salt, began to discern the presence of a sulphur-containing six-membered ring in the molecule. From then on both chemical and X-ray studies made rapid progress and placed structure XVII on a firm basis. New degradation products of cephalosporin C, obtained after hydrogenolysis with Raney nickel, included γ-hydroxyvaline (XIX) and α-hydroxy-β-methylbutenolide (XX). One of the sulphur-containing products formed on acid hydrolysis of cephalosporin C appeared to have the structure XXI (10).



XIX



XX

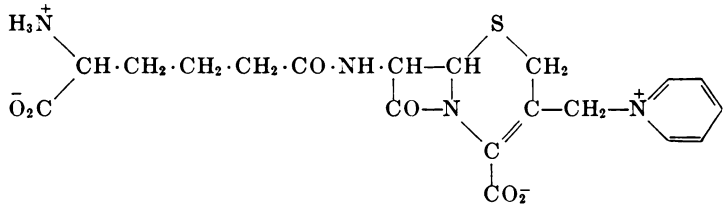


XXI

The X-ray crystallographic analysis enabled the positions of all the atoms in the molecule to be determined (57). It showed that the hydrogen atoms attached to the β-lactam ring are *cis*, as they are in the penicillins.

3. Reaction of cephalosporin C with heterocyclic bases. Cephalosporin C, unlike penicillin N, reacted with pyridine in neutral aqueous solution to yield a new compound with antibacterial activity which showed no net charge on electrophoresis at pH 5 to 7 and was named cephalosporin C_A (pyridine). The properties

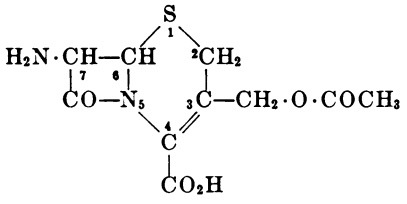
of this compound indicated that the allylic acetoxy group in cephalosporin had been replaced by a pyridinium ion to give the structure XXII. Analogous quaternary compounds were formed when cephalosporin C reacted with a



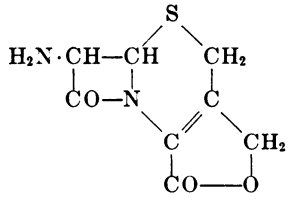
XXII

number of substituted pyridines, including sulphapyridine, and with sulphathiazole (52). Deacetylcephalosporin C, as was to be expected, did not react with pyridine (65).

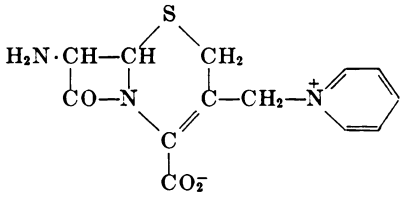
4. 7-Aminocephalosporanic acid (7-ACA) and its derivatives. Cephalosporin C was much more stable than penicillin N in aqueous solution at pH 2 to 3 and did not isomerize to yield a compound of the penillic acid type. In solutions of lower pH one of the first products to be formed was deacetylcephalosporin C lactone (XVIII), but, together with the latter, there appeared small amounts of compounds formed by hydrolytic removal of the α-aminoadipoyl side-chain from cephalosporin C, including the cephalosporin C nucleus (XXIII) and its corresponding deacetyl lactone (XXIV) (70). The nucleus (XXIII) was named 7-aminocephalosporanic acid (7-ACA) since its relationship to cephalosporin C was similar to that of 6-aminopenicillanic acid (6-APA) to penicillin N. A pyridin-



XXIII



XXIV



XXV

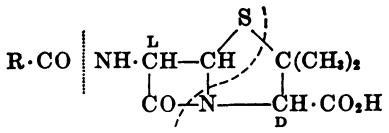
ium derivative of 7-ACA (XXV) was obtained by hydrolysis of cephalosporin C_A (pyridine) with dilute acid at room temperature or by treatment of 7-ACA with aqueous pyridine.

Treatment of 7-ACA with acid chlorides in aqueous sodium bicarbonate resulted in the acylation of the 7-amino group. In this way it was possible to

obtain a new series of compounds containing the nucleus of cephalosporin C but different side-chains. Thus 7-phenacetamidocephalosporanic acid, prepared from 7-ACA and phenylacetyl chloride has a structural relationship to benzylpenicillin (6-phenacetamidopenicillanic acid) which is similar to that of cephalosporin C to penicillin N (70).

C. Biosynthesis

1. Biosynthesis of penicillin N and other penicillins. The structure of penicillin N, like that of other penicillins, can be dissected into residues of D-valine, L-cysteine, and a carboxylic acid side-chain (RCO_2H) as shown by the broken lines in XXVI. Studies by Arnstein and by Stevens and their colleagues with



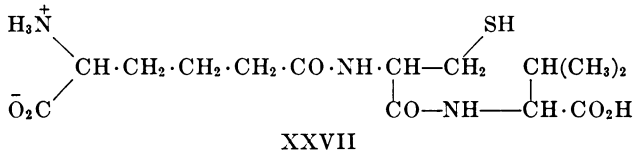
XXVI

labelled precursors have shown that L-valine, L-cysteine, and phenylacetic acid are incorporated into benzylpenicillin (R = $C_6H_5CH_2$) by *Penicillium chrysogenum*. L-Valine undergoes a change in configuration at its α-carbon atom (to yield a D-penicillamine residue) during the biosynthesis of the thiazolidine ring. This change probably occurs through the formation of an α,β-dehydrovaline residue and the subsequent addition of sulphur to the double bond (15, 42). In the absence of an added side-chain precursor, 6-aminopenicillanic acid (6-APA, VIII) accumulates in fermentations with *P. chrysogenum* (21). 6-Aminopenicillanic acid also appears to be formed by *Emericellopsis minima*, *Cephalosporium salmosynnematum*, and *Cephalosporium* sp. C.M.I. 49,137, in some media, together with penicillin N. Whether the N-acylation of 6-APA represents a final step in the biosynthesis of the penicillins is uncertain. Penicillin amidases occur widely in micro-organisms and can bring about the formation of 6-APA from a number of different penicillins (36, 59, 93). However, although *E. minima* produced an amidase which removed the side-chain of penicillin V (phenoxymethylpenicillin), this enzyme had no effect on penicillin N (21).

There is no reason to doubt that L-valine and L-cysteine are used for the biosynthesis of penicillin N, as they are for that of benzylpenicillin. The question then arises why the only penicillin which appears to be synthesized by certain species of the genus *Cephalosporium* or *Emericellopsis* is one with a side-chain derived from D-α-aminoadipic acid, while *P. chrysogenum* can synthesize penicillins with non-polar side-chains derived from a variety of acids, such as phenylacetic and phenoxyacetic, when the appropriate acid is added to the culture medium as a precursor. α-Aminoadipic acid is one of the less common naturally occurring amino acids. It has been isolated, in an unstated optical configuration, from *Vibrio cholerae* (28). L-α-Aminoadipic acid occurs in certain plants (23, 108), *Aspergillus oryzae* (108), and *Penicillium chrysogenum* (17) and has been impli-

cated in the metabolism of lysine in *Neurospora crassa* (72, 110), the guinea pig (30), and man (31). But D-α-aminoadipic acid appears to have been obtained, hitherto, only from penicillin N and cephalosporin C. Biosynthesis of both the L- and D- forms of the amino acid may involve condensation of acetyl coenzyme A with α-ketoglutarate to yield α-ketoadipate by a series of reactions analogous to those by which α-ketoglutarate is formed from acetyl coenzyme A and oxaloacetate in the citric acid cycle (102, 105).

An indication that α-aminoadipic acid plays a role in the biosynthesis of penicillins other than penicillin N came from the finding of Arnstein *et al.* (16) that the tripeptide δ-(α-aminoadipoyl)cysteinylvaline (XXVII), or the corresponding disulphide, was present in the mycelium of *P. chrysogenum*. This tripeptide shows a formal resemblance to glutathione (γ-glutamylcysteinylglycine) and by analogy with the latter its biosynthesis would be expected to involve the coupling of α-aminoadipic acid with cysteine followed by the coupling of the resulting dipeptide with valine.



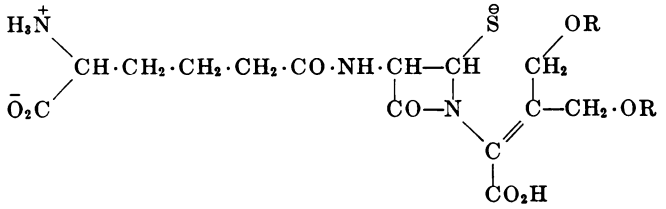
XXVII

Arnstein and Morris (17) suggested that the tripeptide XXVII might be an intermediate in the biosynthesis of benzylpenicillin, the α-aminoadipic acid residue being exchanged for a residue of phenylacetic acid, under the action of a transferase, at some stage of the process. This provided a possible explanation of the finding that isotopically labelled L-cystinyl-L-valine was a relatively inefficient precursor of benzylpenicillin, since L-cysteinyl-L-valine might not be on the main biosynthetic pathway to the tripeptide. The absence of an appropriate transferase might account for the fact that penicillin N, but no other penicillin, has been obtained from species of *Cephalosporium* or *Emericellopsis*. In this connection it may be significant that α-aminoadipic acid isolated from the mycelium of *P. chrysogenum* was preponderantly the L-form, while that in penicillin N is the D-form. It has been reported that *P. chrysogenum* metabolises L-α-aminoadipic acid but not the D-isomer (98).

Further evidence that α-aminoadipic acid is involved in the biosynthesis of benzylpenicillin, as well as of penicillin N, was obtained by Somerson *et al.* (100). They found that DL-α-aminoadipic acid stimulated the synthesis of benzylpenicillin by resting cells of *P. chrysogenum* in a synthetic medium. In a complex medium, allowing growth of the fungus, penicillin production was inhibited by lysine and this inhibition was reversed by DL-α-aminoadipic acid. It seemed possible that lysine inhibited the formation of α-aminoadipic acid or the incorporation of the latter into δ-(α-aminoadipoyl)cysteinylvaline.

2. Biosynthesis of cephalosporin C. The structural relationships of penicillin N and cephalosporin C suggest that the two substances are synthesised from similar units by pathways which have features in common. Thus C-6, C-7, C-8, and the sulphur atom of cephalosporin C (XVII) could be derived from cysteine, while

C-2, C-3, C-4, C-9, and C-10 could come from a valine residue which was raised to a higher level of oxidation at some stage of the biosynthetic process. γ-Hydroxyvaline, which could also be formed by the oxidation of valine or one of its precursors, has been found to be present in certain plants (87). Thus a final step in the biosynthesis of cephalosporin C might be ring closure of an intermediate such as XXVIII.



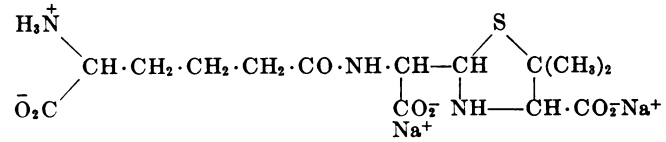
XXVIII

An interesting problem is raised by the ability of methionine, and in particular the D-isomer, to increase the production of penicillin N and cephalosporin C. Demain and Newkirk (43) have suggested that methionine may repress its own biosynthesis, as it does in *E. coli* (96), by inhibiting the formation of cystathionase, and that this may help to maintain cysteine at an optimum concentration in the cell for the synthesis of penicillin N and cephalosporin C. Cystathionase, in addition to its action on cystathionine, appears to degrade cysteine to pyruvate.

Despite the progress that has been made, our views on the details of the reaction sequences leading to the formation of penicillin N and cephalosporin C are still largely in a speculative stage. The interpretation of the results of experiments in this field is likely to be complicated by the existence of permeability barriers between the cell and the medium, and an important step forward will have been made when some of the reactions concerned can be studied in cell-free systems.

D. Derivatives of 6-APA and 7-ACA as substrates, inhibitors, and inducers of penicillinase

1. Maximum rates of hydrolysis. Cephalosporin C (the δ-(D-α-aminoadipoyl) derivative of 7-ACA, XXIII) was found to be very much less susceptible than penicillin N (the corresponding derivative of 6-APA, VIII) to inactivation by the enzyme penicillinase (1, 6, 7, 79). A similar difference in sensitivity to penicillinase was subsequently observed between other N-acyl derivatives of 7-ACA and the corresponding derivatives of 6-APA (41). During the course of work in this field, however, it became clear that penicillinase produced by strains of *B. cereus* differed strikingly, in some respects, from that produced by strains of *Staph. aureus* (11).



XXIX

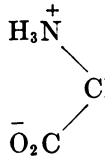
With a given concentration of penicillinase from either *B. cereus* 569 or a strain of *Staph. aureus* the maximum rate of hydrolysis (Vmax.) of penicillin N to its corresponding penicilloate (XXIX) is about half that of benzylpenicillin. In contrast, Vmax. for cephalosporin C is only about 10^{-4} times that for benzylpenicillin with purified penicillinase from *B. cereus* 569 and less than 10^{-3} times that for benzylpenicillin with the crude enzyme from a strain of *Staph. aureus*. A similar resistance to hydrolysis by these penicillinases is shown by the phenylacetyl derivative of 7-ACA (41). Deacetylcephalosporin C is even more resistant to hydrolysis than cephalosporin C itself. On the other hand, Vmax. for cephalosporin C_A (pyridine) (XXII) is greater than that for cephalosporin C, and Vmax. for deacetylcephalosporin C lactone (XVIII) is of the same order as that of benzylpenicillin. The exceptional behaviour of the lactone may be correlated with the fact that the β-lactam ring of this substance is much less stable in aqueous solution at pH 7 in the absence of penicillinase than is the β-lactam ring of either benzylpenicillin or cephalosporin C.

2. Competitive inhibition. Although cephalosporin C is highly resistant to hydrolysis by penicillinase from *B. cereus*, it is a competitive inhibitor of the action of the enzyme on benzylpenicillin. If E represents the enzyme concentration, S the substrate concentration, P the product of the reaction, I the inhibitor concentration, Km the Michaelis content of the enzyme-substrate system, and Ki the dissociation constant of the enzyme inhibitor complex, then according to the simple Michaelis-Menten equation

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad \text{and} \quad K_m = \frac{k_2 + k_3}{k_1}$$

while $$E + I \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EI \quad \text{and} \quad K_I = \frac{k_2'}{k_1'}.$$

With cephalosporin C as inhibitor and benzylpenicillin as substrate, the value of Ki/Km was found to be close to 3. Similar values of Ki/Km were obtained with the phenylacetyl and other derivatives of 7-ACA as inhibitor or with penicillin N as substrate. These results indicate that the affinity for the enzyme of a number of different N-acyl derivatives of 7-ACA is of the same order as that of the corresponding derivatives of 6-APA, and that the affinity is not greatly affected by considerable changes in the nature of the N-acyl residue.

In contrast to its effect on the enzyme from *B. cereus*, cephalosporin C has no significant inhibitory effect on the hydrolysis of benzylpenicillin by a staphylococcal penicillinase. The phenylacetyl derivative of 7-ACA, however, is a powerful competitive inhibitor of the staphylococcal enzyme, Ki/Km being 1.7×10^{-2} with benzylpenicillin as substrate and 0.8×10^{-3} with penicillin N as substrate. It thus appears that the affinities of N-acyl derivative of 6-APA and 7-ACA for penicillinase from *Staph. aureus* are greatly increased when the acyl residue is changed from  to $C_6H_5CH_2\cdot$ (41). The value

of Km for benzylpenicillin with a staphylococcal enzyme has been reported to be 2.5×10^{-6} M (80), whereas the value with penicillinase from *B. cereus* is about 4.9×10^{-5} M. On the other hand Km for penicillin N is about 5×10^{-5} M with either enzyme.

Although very high concentrations of penicillinase from *B. cereus* are able to catalyse the hydrolysis of cephalosporin C and the phenylacetyl derivative of 7-ACA at a measurable rate, the rate has been found to decline after relatively short contact between enzyme and substrate. It appears that these compounds, in addition to acting as competitive inhibitors, are able to inhibit the enzyme in a non-competitive manner (41).

3. Cephalosporinase. Relative to benzylpenicillin, cephalosporin C and deacetylcephalosporin C are more susceptible to hydrolysis by culture filtrates of *B. cereus* than by highly purified penicillinase obtained from these culture filtrates. It appears that *B. cereus* produces a cephalosporinase to which the β-lactam ring of cephalosporin C and some of its derivatives is less resistant than it is to the enzyme responsible for most of the penicillinase activity in purified preparations (41). Whether the cephalosporinase and purified penicillinase represent different conformations of a single protein remains to be determined.

4. Enzyme induction. Like the penicillins, cephalosporin C and other derivatives of 7-ACA induce the synthesis of penicillinase by *B. cereus* 569. In optimum concentration cephalosporin C is a more powerful inducer than benzylpenicillin, but the optimum concentration is much higher for cephalosporin C (about 100 μg/ml) than for benzylpenicillin (about 1 μg/ml) or for the phenylacetyl derivative of 7-ACA (about 1 μg/ml) (41, 89). In contrast, the concentration of benzylpenicillin required to induce the synthesis of penicillinase at a given rate by a strain of *Staph. aureus* is higher than the corresponding concentration of cephalosporin C and much higher than that of the phenylacetyl derivative of 7-ACA. The apparent efficiency of the derivatives of 7-ACA as inducers of the staphylococcal enzyme may be attributed, at least in part, to their stability in the presence of the enzyme. The induction of staphylococcal penicillinase appears to require the continued presence of the inducer in the medium surrounding the cells. The induction of penicillinase by benzylpenicillin in *B. cereus* 569 is unusual in that brief contact of the inducer with the cells enables the latter to synthesize enzyme for a considerable time at a constant rate (88).

E. Antibacterial properties

1. Activity of penicillin N. Penicillin N is much less active than many other penicillins against gram-positive bacteria. For example, its activity against certain strains of *Staph. aureus*, when measured by the serial dilution method, is less than 1% of that of benzylpenicillin. A strain of *Staph. aureus* which had acquired an increased resistance to benzylpenicillin *in vitro*, by subculture in the presence of the drug, was found to be more resistant to penicillin N than the parent strain. Unlike benzylpenicillin, however, penicillin N is at least as active against many gram-negative bacteria as it is against gram-positive bacteria. In consequence, its activity against a number of gram-negative organisms

is several-fold that of benzylpenicillin, and against some of the salmonellae more than ten-fold (4, 54, 56, 61, 81).

The striking influence on antibacterial activity of the positively charged amino group in the side-chain of penicillin N is illustrated by the changes which accompany acylation of this group. N-Benzoyl penicillin N is several times as active as penicillin N itself against *Staph. aureus*, and about one tenth as active against *Salm. typhi*.

2. Activity of cephalosporin C and other derivatives of 7-ACA. Cephalosporin C resembles penicillin N in showing a wide range of activity, but with many bacteria, including the salmonellae and penicillin-sensitive strains of *Staph. aureus*, the minimum concentration at which it inhibits growth *in vitro* is about ten times that of penicillin N. Deacetylcephalosporin C and the corresponding lactone are less active than cephalosporin C, and the activity of 7-ACA is very low. As with penicillin N, acylation of the amino group of cephalosporin C is accompanied by an increase of several-fold in activity against *Staph. aureus*, and a decrease in activity against *Salm. typhi*.

Cephalosporin C_A (pyridine) (XXII) is more than ten times as active as cephalosporin C against many strains of penicillin-sensitive and penicillin-resistant staphylococci, but shows an activity similar to that of cephalosporin C against *Salm. typhi* (52, 62). Staphylococci which had acquired resistance to cephalosporin C_A (pyridine) by subculture in the presence of the antibiotic were reported to have an increased resistance to cephalosporin C but not to benzylpenicillin. The resistance thus acquired decreased on subculture of the organism in normal medium (62).

A particularly striking change in antibacterial activity accompanies the replacement of the α-aminoadipoyl group of cephalosporin C (XVII) by a phenylacetyl group or group of similar structure. Thus the phenylacetyl derivative of 7-ACA is several hundred times as active as cephalosporin C against certain strains of *Staph. aureus*, showing an activity which is of the same order of magnitude as that of benzylpenicillin. Cephalosporin C and the phenylacetyl derivative of 7-ACA show activities against penicillinase-producing strains of staphylococci which are similar to their activities against strains which do not produce penicillinase (45).

The ability of these compounds to maintain their activity against the penicillinase-producing strains is due largely to the very low values for their maximum rates of hydrolysis by the enzyme. In this they differ from 2:6-dimethoxyphenylpenicillin (methicillin), the activity of which against the penicillinase-producing staphylococci is determined, to a considerable extent, by its very low affinity for the enzyme (80, 94). Cephalosporin C and benzylpenicillin were found to act synergistically when used together *in vitro* against one penicillinase-producing strain of *Staph. aureus* (40). It was at first thought that the synergism might be attributed to a competitive inhibition by cephalosporin C of the enzymic inactivation of the penicillin; but the subsequent finding that cephalosporin C does not compete effectively with benzylpenicillin for extracellular penicillinase from *Staph. aureus*, as it does for penicillinase from *B. cereus*, indicated that this

hypothesis might become untenable (11, 41). With other penicillinase-producing strains of *Staph. aureus* no significant synergism has been observed (73). Experiments with the N-phenylacetyl derivative of 7-ACA have shown that only a very powerful competitive inhibitor is likely to give sufficient protection to benzylpenicillin from staphylococcal penicillinase to exert a marked synergistic effect (41). However, synergism between derivatives of 7-ACA and 6-APA might arise from non-competitive, as well as competitive, inhibition.

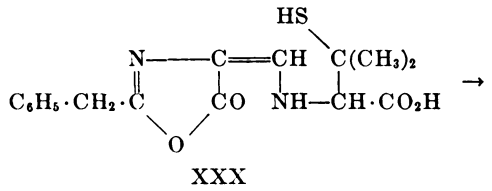
3. Mode of action. Penicillin N and cephalosporin C exert a rapid bactericidal effect on growing sensitive organisms. Like benzylpenicillin, they appear to interfere with the synthesis of the bacterial cell wall, for they bring about the lysis of growing staphylococci and lysis is preceded by the accumulation in the cells of nucleotides containing uridine-5′-pyrophosphate and the N-acetyl derivative of 3-O-α-carboxyethyl-D-glucosamine (muramic acid) (9). In the main nucleotide, muramic acid is linked to the peptide L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine. Muramic acid and the amino acids of the peptide occur in the cell wall of *Staph. aureus* in the same proportions as they do in the nucleotide. It has thus been suggested that the nucleotide mediates the incorporation of the N-acetylmuramic acid peptide into new cell wall and that the nucleotide accumulates when this process is blocked (85). A strain of *Vibrio cholerae* used for the assay of cephalosporin C has been found to form spheroplasts in subinhibitory concentrations of the antibiotic (29).

F. Pharmacological and chemotherapeutic properties

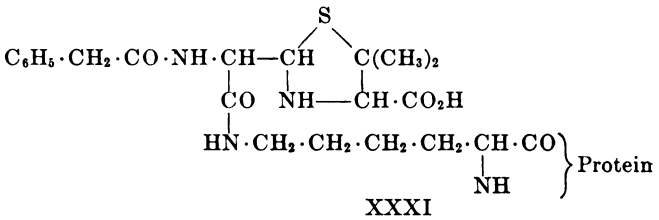
1. Pharmacological properties of penicillin N. Heatley and Florey (54) found that penicillin N sodium salt was even less toxic than sodium benzylpenicillin when given intravenously to mice and that it was also less toxic than benzylpenicillin when given intracisternally to rabbits. They also found that penicillin N was excreted somewhat less rapidly in the urine than benzylpenicillin when given intravenously to rats and that it was absorbed less readily from the intestine. These findings have been confirmed and extended by others. The acute intravenous toxic dose of sodium penicillin N in rats approaches that of sodium chloride (60). Like benzylpenicillin, however, penicillin N is much more toxic to the guinea pig than to other species. Penicillin N appears to diffuse readily into the extracellular space but not into the cerebrospinal fluid. It is excreted in the dog mainly by glomerular filtration and is not concentrated by the gall bladder. In man it is poorly absorbed from the gastrointestinal tract but well absorbed after intramuscular injection.

Several patients who were sensitive to benzylpenicillin and a number of other penicillins were reported to give no reaction on intradermal injection of penicillin N (25, 45); the use of a passive transfer technique appeared to show that serum reagins to benzylpenicillin or phenoxymethylpenicillin did not react with penicillin N (24). These results suggested that the side-chain of the penicillin molecule might play a role as an antigenic determinant. They were consistent with the hypothesis of Levine and Ovary (68) that the antigenic determinants responsible for hypersensitivity to benzylpenicillin are produced by reaction of D-benzyl-

penicillenic acid (XXX), a transformation product of the penicillin, with the ϵ-amino groups in the lysine residues of tissue proteins. This reaction leads to the formation of conjugated proteins containing ϵ-N-(D-α-benzylpenicilloyl)lysine groups (XXXI).



XXX



XXXI

Other findings, however, indicate that the final picture may be more complicated and that a variety of different antibodies may be involved. Human erythrocytes which had been sensitized by exposure to either benzylpenicillin or penicillin N were found to be agglutinated by certain sera from a small proportion of patients who had received penicillin therapy (69). Torii and Kohoriuchi (104) reported that a conjugate obtained by incubating benzylpenicillin with egg albumin was able to induce a specific anaphylaxis in guinea pigs, and that benzylpenicillin could not be replaced successfully by benzylpenicillenic acid. They concluded that abnormal protein, with a strong capacity for binding penicillin, was present in the sera of patients who showed anaphylactoid reactions. Stewart (101) has stated that patients sensitized to benzylpenicillin showed cutaneous sensitivity not only to a number of other penicillins but also to 6-aminopenicillanic acid.

2. Therapeutic properties of penicillin N. Olson and Jennings (83) found that penicillin N, given subcutaneously, was able to free mice from otherwise lethal infections with *Salm. typhimurium* and *Salm. typhi* and to free chicks from infection with *Salm. pullorum.* In further experiments penicillin N was shown to protect mice from infection with *E. coli*, *Proteus vulgaris*, and *P. mirabilis* as well as with *Salm. typhimurium*; it was more effective than chloramphenicol, streptomycin, or benzylpenicillin against infections with *Salm. typhimurium* and was the only one of these antibiotics that was capable of controlling all four types of infection. Moreover, two of the organisms against which penicillin N was effective *in vivo*, *E. coli* and *P. vulgaris*, were relatively resistant *in vitro*, and the activity of penicillin N *in vivo* against *Staph. aureus* and *Str. pyogenes*, when compared with that of benzylpenicillin, was greater than would have been expected from its activity *in vitro* (56, 90).

Penicillin N has been used, on a small scale, for the treatment of typhoid

fever in man. From a clinical trial with 15 patients the conclusion was reached that it was an effective drug for the treatment and control of the disease and that it was more effective than chloramphenicol in clearing *Salm. typhi* from the faeces (22). One patient with a severe systemic typhoid infection which had been treated unsuccessfully with benzylpenicillin and chloramphenicol improved rapidly after receiving penicillin N and did not become a carrier (55). Penicillin N has also been used for the treatment of gonorrhoea and syphilis in patients sensitive to benzylpenicillin (25).

3. Pharmacological properties of cephalosporin C and derivatives. Cephalosporin C (XVII), like penicillin N, is remarkably innocuous to the mouse, a dose of 100 mg given intravenously producing no noticeable ill effect (45). It evoked no reaction in patients sensitized to benzylpenicillin who showed cutaneous sensitivity to three other penicillins and to 6-APA (101). Cephalosporin C_A (pyridine) (XXII) may be somewhat less innocuous but its acute toxicity is nevertheless very low. Cephalosporin C and C_A (pyridine) are absorbed into the blood after subcutaneous injection, and after subcutaneous or intravenous injection are excreted almost quantitatively in the urine. They are poorly absorbed, however, from the gastrointestinal tract (62).

4. Therapeutic properties of cephalosporin C and derivatives. Eight 3-hourly doses of 1 mg of cephalosporin C, given subcutaneously, completely protected mice infected intraperitoneally with *Strep. pyogenes*, while all the control animals died within 12 hours (46). Nine doses of 0.125 mg of cephalosporin C_A (pyridine) completely protected mice from a similar infection (62). Doses of 2 mg of cephalosporin C given at 3-hourly intervals completely protected mice from an infection with a penicillinase-producing strain of *Staph. aureus* which did not respond to treatment with benzylpenicillin (46). Smaller doses of the phenylacetyl derivative of 7-ACA gave similar protection (63). It has been reported that the dose of cephalosporin C or cephalosporin C_A (pyridine) required to protect mice which have been infected intraperitoneally with penicillinase-producing staphylococci is reduced by at least 50 to 75% when the cephalosporin is given with an equal amount of benzylpenicillin, although such mice are not protected by benzylpenicillin alone (63).

IV. CONCLUDING REMARKS

The antibiotics that have now been isolated from culture fluids of species of *Cephalosporium* or *Emericellopsis* show a highly selective toxicity and have structures which are novel variants of those of previously well-known groups of natural products.

Cephalosporin P stands out as one of the very few members of the steroid group that are known to have significant antibacterial activity. Its mode of action, like that of the chemically related fungal product, helvolic acid, is still not understood. It comes near to being a useful chemotherapeutic agent for staphylococcal infections, but it is less effective *in vivo*, in comparison with several other antibiotics, than might be expected from its activity *in vitro* and from the ease with which it can be obtained in antibacterial concentration in the blood.

Further advances in the chemistry of cephalosporin P may be necessary before the possibility of improving on its therapeutic activity by structural modifications can be adequately explored. But the successful use in man of the chemically related substance, fucidin, suggests that this is a field worth further investigation.

Penicillin N has the normal penicillin nucleus (6-aminopenicillanic acid), but a polar side-chain of a type not previously encountered. Its discovery revealed that striking and useful changes in antibacterial activity and other biological properties could accompany an appropriate alteration in the side-chain of benzylpenicillin. More recently, the isolation of 6-aminopenicillanic acid has made it possible to produce penicillins not previously accessible, including those with side-chains derived from 2,6-dimethoxybenzoic acid (methicillin) (94), 5-methyl-3-phenylisoxazole-4-carboxylic acid (44), and D-α-phenylglycine (ampicillin, Penbritin) (95), and has opened the way to an extensive exploration of the variation of biological properties with changes in the side-chain.

Penicillin N itself is clinically effective and appears to be superior to chloramphenicol for the treatment of typhoid fever. Indeed, there are indications that it is more effective *in vivo* than would be anticipated from its activity *in vitro*. The reasons why it has not found a place among the antibiotics generally available to the physician are probably mainly commercial. The production of pure penicillin N is relatively difficult and expensive, and the incentive to produce it on a large scale has been insufficient in relation to the magnitude of the problems involved.

Cephalosporin C resembles penicillin N in containing a side-chain derived from D-α-aminoadipic acid but differs from it in that the side-chain is condensed with a β-lactam-dihydrothiazine ring system (7-aminocephalosporanic acid, 7-ACA) instead of with a β-lactam-thiazolidine ring system (6-aminopenicillanic acid, 6-APA). It is clear that cephalosporin C and penicillin N are related biosynthetically, but the details of this relationship remain to be elucidated. There is no evidence that the biosynthesis of α-aminoadipic acid is a rate-limiting process in the formation of penicillin N or cephalosporin C, and the indication that this amino acid is involved in the biosynthesis of penicillins by *Penicillium chrysogenum* needs further exploration. Biochemical problems also arise in connection with the sulphur metabolism of *Cephalosporium* and *Emericellopsis* sp. and the stimulation of their ability to produce penicillin N and cephalosporin C by D-methionine (27, 43).

The N-acyl derivatives of 7-ACA appear to be as innocuous to animals as the corresponding derivatives of 6-APA, but to be from five to ten times less active than the latter against a number of bacteria *in vitro* and, in some cases at least, to be less readily absorbed from the gastrointestinal tract. Certain changes in the N-acyl group, such as a change from α-aminoadipoyl to phenylacetyl, are accompanied by striking changes in the antibacterial activity of derivatives of 7-ACA and in the affinity of these derivatives for staphylococcal penicillinase. Similar differences in properties are found among the corresponding N-acyl derivatives of 6-APA. The various derivatives of 7-ACA and 6-APA appear to have essentially similar modes of action. Some of the large differences in their

antibacterial activities may perhaps be attributed to an influence of the side-chain on the ease with which they reach, or combine with, a specific receptor in the cell (2, 89). It has been reported that penicillin N is bound at a much slower rate than benzylpenicillin by resting cells of *Staph. aureus* (74).

Despite the similarities between the two families of compounds, the N-acyl derivatives of 7-ACA are sharply distinguished from the corresponding derivatives of 6-APA (penicillins) by certain properties that are conferred on them by the β-lactam-dihydrothiazine ring system. The derivatives of 7-ACA are relatively stable in dilute acid, and they are highly resistant to hydrolysis by purified penicillinase from *B. cereus*, or penicillinase from *Staph. aureus*, irrespective of the nature of their side-chains and their affinity for the enzyme. The activities of 2:6-dimethoxyphenylpenicillin and the N-phenylacetyl derivative of 7-ACA against penicillinase-producing strains of *Staph. aureus* appear to depend mainly on two different properties. The former substance has a low affinity for the enzyme, and its rate of hydrolysis is far below the maximum at the minimum concentrations in which it shows antibacterial activity, while the latter substance has a high affinity but also a very low rate of hydrolysis in concentrations sufficient to saturate the enzyme.

The penicillinase-producing strains of *Staph. aureus* raised a serious clinical problem because these strains survived and spread in hospitals when other strains were suppressed by the use of the natural penicillins. This problem was magnified by the ability of the staphylococcus to acquire resistance to antibiotics of other types with great facility. Thus the production of new penicillins or penicillin-like compounds which were resistant to penicillinase became an objective of some practical importance. 2:6-Dimethoxyphenylpenicillin and an isoxazolyl derivative of 6-APA have already been shown to be clinically valuable. The properties of some of the N-acyl derivatives of 7-ACA, including their very low toxicity and their resistance to penicillinase, suggest that this group of substances may also provide useful additions to the drugs now available for the treatment of staphylococcal infections. The fact that organisms which do not produce penicillinase have retained their sensitivity to benzylpenicillin during its continued use in medicine provides ground for hope that the new compounds will not rapidly lose their usefulness. But it would be unwise to assume that any single derivative of 7-ACA or 6-APA will provide a permanent solution to the staphylococcal problem. Penicillinases from different organisms are known to differ in their enzymic properties, and it is conceivable that strains of *Staph. aureus* will eventually emerge which will produce penicillinases with new specificities. The discovery of the penicillin amidases has indicated that the amide linkage of the side-chain, as well as that of the β-lactam ring, is a potential source of weakness in some N-acyl derivatives of 6-APA and 7-ACA. Moreover, although it is the penicillinase-producing strains of *Staph. aureus* which have hitherto restricted the value of benzylpenicillin for the treatment of staphylococcal infections, the possibility remains that new pathogenic strains will appear with a resistance to penicillins and related compounds which depends on factors other than an ability to produce penicillinase (19).

Penicillinase is produced not only by staphylococci and gram-positive bacilli but also by gram-negative bacilli. The resistance of certain strains of *E. coli*, *Pr. vulgaris*, and *A. aerogenes* to the penicillinase-sensitive, "broad spectrum," 6[D(−)α-aminophenylacetamido]penicillin (Penbritin) may be due to the destruction of the antibiotic by penicillinase produced by these organisms (95). It remains to be seen whether some of the penicillinase-resistant derivatives of 7-ACA will prove useful for the treatment of infections with such gram-negative bacteria.

The production of new drugs that are effective against penicillinase-producing bacteria may not represent the only outcome of value to medicine from further work in this field. The isolation of 6-APA and 7-ACA has enabled detailed studies to be made, among the N-acyl derivatives of these compounds, of relationships between chemical structure and biological properties. In 7-ACA, the acetoxymethyl group attached to the dihydrothiazine ring, as well as the 7-amino group, provides a modifiable centre from which new series of active substances may be accessible. Properties known to be variable, in some cases independently, among 6-APA or 7-ACA derivatives, include range of antibacterial activity *in vitro* and *in vivo*, efficiency of absorption when given by mouth, and ability to evoke hypersensitivity reactions, as well as affinity for penicillinase, rate of hydrolysis by penicillinase, and ability to induce the formation of penicillinase. The results of current investigations are likely to make interesting additions to what is now known about the influence of structural changes on such properties. But some time may elapse before the extent of their impact on chemotherapy can be adequately assessed.

REFERENCES

1. Abraham, E. P.: New penicillins and other antibiotics containing nitrogen and sulphur. Giorn. Microbiol. **2**: 102–115, 1956.
2. Abraham, E. P.: Biochemistry of Some Peptide and Steroid Antibiotics. John Wiley & Sons, Inc., New York, 1957.
3. Abraham, E. P., Newton, G. G. F., Crawford, K., Burton, H. S. and Hale, C. W.: Cephalosporin N: a new type of penicillin. Nature, Lond. **171**: 343, 1953.
4. Abraham, E. P., Newton, G. G. F. and Hale, C. W.: Isolation and some properties of cephalosporin N, a new penicillin. Biochem. J. **58**: 94–102, 1954.
5. Abraham, E. P. and Newton, G. G. F.: Synthesis of D-α-amino-α-carboxyvalerylglycine (a degradation product of cephalosporin N) and of DL-α-amino-α-carboxyvaleramide. Biochem. J. **58**: 266–268, 1954.
6. Abraham, E. P. and Newton, G. G. F.: Experiments on the degradation of cephalosporin C. Biochem. J. **62**: 658–665, 1956.
7. Abraham, E. P. and Newton, G. G. F.: Comparison of the action of penicillinase on benzylpenicillin and cephalosporin N and the competitive inhibition of penicillinase by cephalosporin C. Biochem. J. **63**: 628–634, 1956.
8. Abraham, E. P. and Newton, G. G. F.: Chemistry of the cephalosporins. Chem. Soc. Lond., Special Publication No. 5, 97–107, 1956.
9. Abraham, E. P. and Newton, G. G. F.: Structure and function of some sulphur-containing peptides. In: Amino Acids and Peptides with Antimetabolic Activity, Ciba Foundation Symposium ed. by G. E. W. Wolstenholme and C. M. O'Connor, pp. 205–223. J. & A. Churchill Ltd., 1958.
10. Abraham, E. P. and Newton, G. G. F.: The structure of cephalosporin C. Biochem. J. **79**: 377–393, 1961.
11. Abraham, E. P. and Newton, G. G. F.: New penicillins, cephalosporin C and penicillinase. Endeavour **20**: 92–100, 1961.
12. Abraham, E. P., Newton, G. G. F., Schenck, J. R., Hargie, M. P., Olson, B. H., Schuurmans, D. M., Fisher, M. W. and Fusari, S. A.: Identity of cephalosporin N and synnematin B. Nature, Lond. **176**: 551, 1955.
13. Alicino, J. F.: Iodometric assay of natural and synthetic penicillins, 6-aminopenicillanic acid and cephalosporin C. Analyt. Chem. **33**: 648–649, 1691.
14. Allinger, N. L. and Coke, J. L.: The structure of helvolic acid. III. J. org. Chem. **26**: 4522–4529, 1961.

15 ARNSTEIN, H. R. V.: The biosynthesis of penicillin and some other antibiotics. Annu. Rep. chem. Soc. **54:** 339–352, 1957.
16. ARNSTEIN, H. R. V., MORRIS, D. AND TOMS, E. J.: Isolation of a tripeptide containing α-aminoadipic acid from the mycelium of *Penicillium chrysogenum* and its possible significance in penicillin biosynthesis. Biochim. biophys. Acta **35:** 561–562, 1959.
17. ARNSTEIN, H. R. V. AND MORRIS, D.: The structure of a peptide, containing α-aminoadipic acid, cysteine and valine, present in the mycelium of *Penicillium chrysogenum*. Biochem. J. **76:** 357–361, 1960.
18. BAIRD, B. M., HALSALL, T. G., JONES, E. R. H. AND LOWE, G.: Cephalosporin P_1. Proc. chem. Soc., 259, 1961.
19. BARBER, M.: Methicillin-resistant staphylococci. J. clin. Path. **14:** 385–393, 1961.
19a. BARBER, M. AND WATERWORTH, P. M.: Antibacterial activity *in vitro* of fucidin. Lancet **1:** 931–932, 1962.
20. BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C. AND ROLINSON, G. N.: Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature, Lond. **183:** 257–258, 1959.
21. BATCHELOR, F. R., CHAIN, E. B. AND ROLINSON, G. N.: 6-Aminopenicillanic acid. 6-Aminopenicillanic acid in penicillin fermentations. Proc. roy. Soc., ser. B **154:** 478–489, 1961.
22. BENAVIDES, L., OLSON, B. H., VARELA, G. AND HOLT, S. H.: Treatment of typhoid with synnematin B. J. Amer. med. Ass. **157:** 989–994, 1955.
23. BERG, A. M., KARI, S., ALFTHAN, M. AND VIRTANEN, A. I.: Homoserine and α-aminoadipic acid in green plants. Acta chem. scand. **8:** 358, 1954.
24. BERRYMAN, G. H. AND FISHERMAN, E. W.: On modification of penicillin antigenicity. J. Allergy **30:** 329–336, 1959.
25. BERRYMAN, G. H. AND SYLVESTER, J. C.: Influence of chemical structure upon biological properties of penicillin. Antibiotics Annual, pp. 521–525, 1959–1960, Medical Encyclopedia, Inc., N. Y.
26. BHUYAN, B. K. AND JOHNSON, M. J.: Chemically defined media for synnematin production. J. Bact. **76:** 376–384, 1958.
27. BHUYAN, B. K., MOHBERG, J. AND JOHNSON, M. J.: Sulphur metabolism of synnematin-producing molds. J. Bact. **76:** 393–399, 1958.
28. BLASS, J. AND MACHEBOEUF, M.: Sur la présence d'aminoacides nouveaux dans les vibrions choleriques: Acide aminoadipique et acide hydroxyaminoadipique. Helv. chim. acta **29:** 1315–1317, 1946.
29. BOND, J. M., BRIMBLECOMBE, R. W. AND CODNER, R. C.: Biological assay of cephalosporin C. J. gen. Microbiol. **27:** 11–19, 1962.
30. BORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, I. AND LOWY, P. H.: The degradation of L-lysine in guinea pig liver homogenate: formation of α-aminoadipic acid. J. biol. Chem. **176:** 1383–1393, 1948.
31. BOULANGER, P. AND BISERTE, G.: The presence of α-aminoadipic acid in urine. Compt. rend. Soc. Biol., Paris **232:** 1451–1453, 1951.
32. BROTZU, G.: Richerche su di un nuovo antibiotico. Lav. Ist. Ig. Cagliari, 1948.
33. BURTON, H. S. AND ABRAHAM, E. P.: Isolation of antibiotics from a species of *Cephalosporium*. Cephalosporins P_1, P_2, P_3, P_4 and P_5. Biochem. J. **50:** 168–174, 1951.
34. BURTON, H. S., ABRAHAM, E. P. AND CARDWELL, H. M. E.: Cephalosporin P_1 and helvolic acid. Biochem. J. **62:** 171–176, 1956.
35. CHAIN, E., FLOREY, H. W., JENNINGS, M. A. AND WILLIAMS, T. I.: Helvolic acid, an antibiotic produced by *Aspergillus fumigatus* mut. *helvola* Yuill. Brit. J. exp. Path. **24:** 108–119, 1943.
36. CLARIDGE, C. A., GOUREVITCH, A. AND LEIN, J.: Bacterial penicillin amidase. Nature, Lond. **187:** 237–238, 1960.
37. CLARK, R. K., FRICKE, H. H. AND LANIUS, B.: The purification of synnematin B by ion-exchange resins. Antibiotics Annual, pp. 749–754, 1956–1957, Medical Encyclopedia, Inc., N.Y.
38. CRAM, D. J. AND ALLINGER, N. L.: Mould metabolites. VIII. Contributions to the elucidation of the structure of helvolic acid. J. Amer. chem. Soc. **78:** 5275–5284, 1956.
39. CRAWFORD, K., HEATLEY, N. G., BOYD, P. F., HALE, C. W., KELLY, B. K., MILLER, G. A. AND SMITH, N.: Antibiotic production by a species of *Cephalosporium*. J. gen. Microbiol. **6:** 47–59, 1952.
40. CRAWFORD, K. AND ABRAHAM, E. P.: The synergistic action of benzylpenicillin and cephalosporin C against a penicillinase-producing strain of *Staphylococcus aureus*. J. gen. Microbiol. **16:** 604–613, 1957.
41. CROMPTON, B., JAGO, M., CRAWFORD, K., NEWTON, G. G. F. AND ABRAHAM, E. P.: Behaviour of some derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid as substrates, inhibitors and inducers of penicillinase. Biochem. J. **83:** 52–63, 1962.
42. DEMAIN, A. L.: The mechanism of penicillin biosynthesis. Advanc. appl. Microbiol. **1:** 23–47, 1959.
43. DEMAIN, A. L. AND NEWKIRK, J.: Studies on the biosynthesis of cephalosporin C. Bact. Proc. **48:** 31, 1962.
44. DOYLE, F. P., LONG, A. A. W., NAYLER, J. H. C. AND STOVE, E. R.: New penicillins stable towards both acid and penicillinase. Nature, Lond. **192:** 1183–1184, 1961.
45. FLOREY, H. W.: Antibiotic products of a versatile fungus. Ann. intern. Med. **43:** 480–490, 1955.
46. FLOREY, H. W.: The medical aspects of the development of resistance to antibiotics. Giorn. Microbiol. **2:** 361–370, 1956.
47. FLOREY, H. W., CHAIN, E., HEATLEY, N. G., JENNINGS, M. A., SANDERS, A. G., ABRAHAM, E. P. AND FLOREY, M. E.: Antibiotics: A survey of penicillin, streptomycin, and other antimicrobial substances from fungi, actinomycetes, bacteria, and plants. Oxford Univ. Press, New York, 1949.
48. FLOREY, H. W., ABRAHAM, E. P., NEWTON, G. G. F., BURTON, H. S., KELLY, B. K., HALE, C. W. AND MILLER, G. A.: Improvements related to the production of an antibiotic substance by a *Cephalosporium* species. Brit. patent 745,208, 1956.
49. FUSARI, S. A. AND MACHAMER, H. E.: Isolation and purification of synnematin B. Preparation of crystalline

N, N'-dibenzylethylenediamino N-acetylsynnematin B. Antibiotics Annual 1957–1958, pp. 529–537, Medical Encyclopedia, Inc., N. Y.

50. Gottshall, R. Y., Roberts, J. M., Portwood, L. M. and Jennings, J. C.: Synnematin, an antibiotic produced by *Tilachlidium*. Proc. Soc. exp. Biol., N. Y. **76:** 307–311, 1951.

50a. Godtfredsen, W., Roholt, K. and Tybring, L.: Fucidin, a new orally active antibiotic. Lancet **1:** 928–931, 1962.

51. Grosklags, J. H. and Swift, M. E.: The perfect stage of an antibiotic producing *Cephalosporium*. Mycologia **49:** 305–317, 1957.

52. Hale, C. W., Newton, G. G. F. and Abraham, E. P.: Derivatives of cephalosporin C formed with certain heterocyclic tertiary bases. The cephalosporin C_A family. Biochem. J. **79:** 403–408, 1961.

53. Harvey, C. L. and Olson, B. H.: Chemically defined media supplemented with precursors for synnematin production. Appl. Microbiol. **6:** 276–280, 1958.

54. Heatley, N. G. and Florey, H. W.: A comparison of the biological properties of cephalosporin N and penicillin. Brit. J. Pharmacol. **8:** 252–258, 1953.

55. Henderson, N. D., Garlock, F. C. and Olson, B. H.: Treatment of acute typhoid with synnematin B. J. Amer. med. Ass. **169:** no. 17, 89–92, 1959.

55a. Hilson, G. R. F.: *In vitro* studies of a new antibiotic (fucidin). Lancet **1:** 932–933, 1962.

56. Hobby, G. L., Pikula-Vrabec, D., Daly, J., Sarrocco, G. and Lenert, T. F.: The action of synnematin against artificially induced *Salmonella* infections in mice. Antibiotics Annual, pp. 793–808, 1956–1957, Medical Encyclopedia, Inc., N. Y.

57. Hodgkin, D. and Maslen, E. N.: An X-ray crystallographic analysis of the structure of cephalosporin C. Biochem. J. **79:** 393–402, 1961.

58. Holker, J. S. E., Powell, A. D. G., Robertson, A., Simes, J. J. H., Wright, R. S. and Gascoigne, R. M.: Chemistry of fungi. IX. Structure of eburicoic acid. J. chem. Soc. **1953:** 2422–2429.

59. Huang, H. T., English, A. R., Seto, T. A., Schull, G. M. and Sobin, B. A.: Enzymatic hydrolysis of the side chain of penicillins. J. Amer. chem. Soc. **82:** 3790–3791, 1960.

60. Hwang, K. and Primack, N.: Pharmacologic studies of synnematin B. Antibiotics Annual, p. 755–780, 1956–1957. Medical Encyclopedia, Inc., N. Y.

61. Jackson, G. G., Rubenis, M. and Mellody, M.: Synnematin B and penicillin G: relationship of molecular differences to bacterial sensitivity and resistance. Antibiotics Annual, pp. 740–748, 1956–1957. Medical Encyclopedia, Inc., N. Y.

62. Jago, M. and Heatley, N. G.: Some biological properties of cephalosporin C and a derivative. Brit. J. Pharmacol. **16:** 170–179, 1961.

63. Jago, M.: Unpublished observations.

64. Jeffery, J. d'A., Abraham, E. P. and Newton, G. G. F.: Further degradation products of cephalosporin C. Isolation and synthesis of 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. Biochem. J. **75:** 216–223, 1960.

65. Jeffery, J. d'A., Abraham, E. P. and Newton, G. G. F.: Deacetylcephalosporin C. Biochem. J. **81:** 591–596, 1961.

66. Kavanagh, F., Tunin, D. and Wild, G.: D-Methionine and the biosynthesis of cephalosporin N. Arch. Biochem. Biophys. **77:** 268–274, 1958.

67. Kavanagh, F., Tunin, D. and Wild, G.: Antibiotics formed by species of *Emericellopsis*. Mycologia **50:** 370–372, 1958.

68. Levine, B. B. and Ovary, Z.: Studies on the mechanism of the formation of the penicillin antigen. III. The N-(D-α-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. J. exp. Med. **114:** 875–904, 1961.

69. Ley, A. B., Harris, J. P., Brickley, M., Liles, B., Jack, J. A. and Cahan, A.: Circulating antibody directed against penicillin. Science **127:** 1118–1119, 1958.

70. Loder, B., Newton, G. G. F. and Abraham, E. P.: The cephalosporin C nucleus (7-aminocephalosporanic acid) and some of its derivatives. Biochem. J. **79:** 408–416, 1961.

71. Miller, G. A., Kelly, B. K. and Newton, G. G. F.: Cephalosporin production. British patent 759,624, 1956.

71a. Miller, I. M., Stapley, E. O. and Charet, L.: Production of synnematin B by a member of the genus *Streptomyces*. Bact. Proc. **49:** 32, 1962.

72. Mitchell, H. K. and Houlahan, M. B.: An intermediate in the biosynthesis of lysine in *Neurospora*. J. biol. Chem. **174:** 883–887, 1948.

73. Moat, A. G., Ceci, L. N. and Bondi, A.: Effect of cephalosporin C and various penicillin derivatives on staphylococcal penicillinase and penicillinase-producing staphylococci. Proc. Soc. exp. Biol., N. Y. **107:** 675–677, 1961.

74. Mohberg, J. and Johnson, M. J.: Comparative study of the binding of synnematin and penicillin by bacterial cells. J. Bact. **76:** 385–392, 1958.

75. Newton, G. G. F. and Abraham, E. P.: Isolation of penicillaminic acid and D-α-aminoadipic acid from cephalosporin N. Nature, Lond. **172:** 395, 1953.

76. Newton, G. G. F. and Abraham, E. P.: A new type of penicillin from a species of *Cephalosporium*. VI Congr. int. Microbiol. Roma **1:** 625, 1953.

77. Newton, G. G. F. and Abraham, E. P.: Degradation, structure and some derivatives of cephalosporin N, a new penicillin. Biochem. J. **58:** 94–102, 1954.

78. Newton, G. G. F. and Abraham, E. P.: Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid. Nature, Lond. **175:** 548, 1955.

79. NEWTON, G. G. F. AND ABRAHAM, E. P.: Isolation of cephalosporin C, a penicillin-like antibiotic containing D-α-aminoadipic acid. Biochem. J. **62**: 651–658, 1956.
80. NOVICK, R. P.: Staphylococcal penicillinase and the new penicillins. Biochem. J. **83**: 229–235, 1962.
81. OLARTE, J. AND FIGUEREDO, G.: The sensitivity of *Salmonella typhi* to synnematin B and other antibiotics. Antibiot. & Chemother. **5**: 162–165, 1955.
82. OLSON, B. H., JENNINGS, J. C. AND JUNEK, A. J.: Separation of synnematin into components A and B by paper chromatography. Science **117**: 76–77, 1953.
83. OLSON, B. H. AND JENNINGS, J. C.: Effect of synnematin B treatment of *Salmonella* infections in mice and chicks. Antibiot. & Chemother. **4**: 11–17, 1954.
84. OLSON, B. H., JENNINGS, J. C., PISANO, M. AND JUNEK, A. J.: Production, recovery and purification of synnematins A and B. Antibiot. & Chemother. **4**: 1–10, 1954.
85. PARE, J. T. AND STROMINGER, J. L.: Mode of action of penicillin. Science **125**: 99–101, 1957.
86. PISANO, M., OLSON, B. AND SAN CLEMENTI, C. L.: A chemically defined medium for the growth of *Cephalosporium salmosynnematum*. J. Bact. **68**: 444–449, 1954.
87. POLLARD, J. K., SONDHEIMER, E. AND STEWARD, F. C.: Hydroxyvaline in *Kalanchoe daigremontiana*. Nature, Lond. **182**: 1356–1357, 1958.
88. POLLOCK, M. R.: Penicillinase adaptation in *B. cereus*: adaptive enzyme formation in the absence of free substrate. Brit. J. exp. Path. **31**: 739–753, 1950.
89. POLLOCK, M. R.: The activity and specificity of inducers of penicillinase production in *Bacillus cereus*, strain NRRL 569. Biochem. J. **66**: 419–428, 1957.
90. RICKHER, C. J., DEYOUNG, M., GRUNDY, W. E. AND SYLVESTER, J. C.: Synnematin B, activity in experimental infections of mice. Antibiotics Annual, pp. 786–792, 1956–1957. Medical Encyclopedia, Inc., N. Y.
91. RITCHIE, A. C., SMITH, N. AND FLOREY, H. W.: Some biological properties of cephalosporin P_1. Brit. J. Pharmacol. **6**: 417–429, 1951.
92. ROBERTS, J. M.: Antibiotic substances produced by species of *Cephalosporium* with a description of new species. Mycologia **44**: 292–306, 1952.
93. ROLINSON, G. N., BATCHELOR, F. R., BUTTERWORTH, D., CAMERON-WOOD, J., COLE, M., EUSTACE, G. C., HART, M. V., RICHARDS, M. AND CHAIN, E. B.: Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature, Lond. **187**: 236–237, 1960.
94. ROLINSON, G. N., BATCHELOR, F. R., STEVENS, S., CAMERON-WOOD, J. AND CHAIN, E. P.: Bacteriological studies on a new penicillin—BRL 1241. Lancet **2**: 564–567, 1960.
95. ROLINSON, G. N. AND STEVENS, S.: Microbiological studies on a new broad-spectrum penicillin, "penbritin." Brit. med. J. **2**: 191–196, 1961.
96. ROWBURY, R. J. AND WOODS, D. D.: Repression of cystathionase production in *Escherichia coli*. Biochem. J. **79**: 36P, 1961.
96a. SCOWER, E. F. AND GARROD, L. P.: A case of staphylococcal septicaemia treated with penicillin and fucidin. Lancet **1**: 933–935, 1962.
97. SHEEHAN, J. H., HENRY LOGAN, K. R. AND JOHNSON, D. A.: The synthesis of substituted penicillins and simpler structural analogues. VII. The cyclisation of a penicilloate derivative to methyl phthalimidopenicillanate. J. Amer. chem. Soc. **75**: 3292–3293, 1953.
98. SHOSTAKOVSKII, M. F., RABINOVITCH, M. S., PREOBRAZHENSKAYA, E. V. AND ZYKOVA, G. N.: Investigations on the synthesis of precursors and fragments of antibiotics. Part 1. α-Aminoadipic acid. J. gen. Chem. (U.S.S.R.) **30**(1): 67–71, 1960.
99. SNEATH, P. H. A. AND COLLINS, J. F.: A method for the chromatographic detection of penicillins and related compounds and of penicillinase. Biochem. J. **79**: 512–514, 1961.
100. SOMERSON, N. L., DEMAIN, A. L. AND NUNHEIMER, T. D.: Reversal of lysine inhibition of penicillin production by α-aminoadipic or adipic acid. Arch. Biochem. Biophys. **93**: 238, 1961.
101. STEWART, G. T.: Cross-allergenicity of penicillin C and related substances. Lancet **1**: 509–510, 1962.
102. STRASSMAN, M. AND WEINHOUSE, S.: Biosynthetic pathways. III. The biosynthesis of lysine by *Torulopsis utilis*. J. Amer. chem. Soc. **75**: 1680–1684, 1953.
102a. TAYLOR, G. AND BLOOR, K.: Antistaphylococcal activity of fucidin. Lancet **1**: 935–937, 1962.
103. THOMAS, R.: Colorimetric detection of penicillins and cephalosporins on paper. Nature, Lond. **191**: 1161–1163, 1961.
104. TORII, T. AND KOHORIUCHI, Y.: Antigenicity of penicillin and its relation to albumin binding, Nature, Lond. **192**: 429–431, 1961.
105. TROWN, P. W., ABRAHAM, E. P., NEWTON, G. G. F., HALE, C. W. AND MILLER, G. A.: Incorporation of [1-^{14}C] acetate into cephalosporin C. Biochem. J. **84**: 157–166, 1962.
106. WAGNER, A. F. AND FOLKERS, K.: The organic and biological chemistry of mevalonic acid. Endeavour **20**: 177–187, 1961.
107. WAKSMAN, S. A. AND HORNING, E. S.: Distribution of antagonistic fungi in nature and their antibiotic action. Mycologia **35**: 47–65, 1943.
108. WINDSOR, E.: α-Aminoadipic acid as a constituent of corn protein. J. biol. Chem. **192**: 595–603, 1951.
109. WILLIAMS, T. I.: Some chemical properties of helvolic acid. Biochem. J. **51**: 538–542, 1952.
110. YURA, T. AND VOGEL, H. J.: On the glutamic family and lysine in Neurospora: enzymic formation of glutamic γ-semialdehyde and α-aminoadipic δ-semialdehyde from penta and hexahomoserine. Biochim. biophys. Acta **24**: 648–649, 1957.