E. P. ABRAHAM

Biochemistry of Some Peptide and Steroid Antibiotics

The second volume in the CIBA Lectures in Microbial Biochemistry series.

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BIOCHEMISTRY of SOME PEPTIDE and STEROID ANTIBIOTICS

By E. P. ABRAHAM

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PREFACE

Although intermittent observations on antibiosis can be traced back to the middle years of the nineteenth century, the systematic study of antibiotics which we see today has developed since 1939. In that year Dubos published his first paper on the production of tyrothricin by *Bacillus brevis;* Florey and Chain and their colleagues undertook the investigations which led to the discovery of the chemotherapeutic properties of Fleming's penicillin; and Waksman and others began a study of the antimicrobial products of actinomycetes which resulted in the finding of streptomycin. One of the mainsprings of the widespread work which followed was certainly the hope of obtaining new substances of therapeutic value. There was little reason to suppose that the study of one group of microorganisms would prove more rewarding, from this point of view, than that of another. Nevertheless, with the striking exception of penicillin, most of the antibiotics that have found a permanent, or even temporary, place in medicine have so

far come from actinomycetes. Whether the actinomycetes are a richer source of such substances than other types of microorganisms, or whether their relative productivity is a reflection of the immense industry with which they have been combed, is difficult to say. But it is possible that each seam in this field will become progressively harder to work as its exploration extends, and it would be rash to assume that antibiotics with interesting and useful biological properties do not still remain to be found among the metabolic products of bacteria and fungi.

Several families of antibiotics produced by bacteria or fungi form the subject of these lectures. They include the bacitracins, which are sulfur-containing polypeptides with unusual structures, and two families of cephalosporins, one consisting of peptides related to penicillin and one that appears to belong to the steroid group. Most of them have chemotherapeutic properties, and bacitracin has found a well-established, though limited, clinical use; the cephalosporins have yet to be produced in sufficient quantity for their value in medicine to be properly assessed.

To the biochemist, a new antibiotic may sometimes appear to be an unexciting thing. Its mode of action often remains unknown and, unlike the hormones and coenzymes, it cannot yet be fitted into the dynamic pattern of the living organism. But it assumes a wider biochemical significance if it is shown to be a member of a chemical group of compounds which is itself of obvious biological importance, or if it can be used to throw light on essential features of microbial activity. The bacitracins and cephalosporins are compounds of some interest from this point of view.

To say that recent advances in our knowledge of naturally occurring peptides have been largely dependent on the development of new techniques is now almost a platitude, and it will be evident that some of the work mentioned in

these lectures could not have been done two decades ago. However, the procedures that are available for the resolution of mixtures of peptides still have their limitations, and the powerful methods that have been devised for the determination of amino acid sequences will not necessarily provide a picture of all the linkages in a peptide molecule. Experience with the bacitracins and cephalosporins has illustrated the fact that a new product is always liable to present new problems.

The bacitracins and cephalosporins have been studied in Oxford during the last ten years, but, although they may be described here with the aid of a large number of domestic references, our knowledge of most of them comes from the work of more than one laboratory. Indeed, in the case of the bacitracins the way in which independent studies produced results that could be grouped together has been a frequent stimulus to the investigations. The work with which I have been concerned has been a collaborative effort to which several colleagues, in particular Dr. G. G. F. Newton, have made indispensable contributions; and it has been facilitated by the stimulating support of Sir Howard Florey and by help from the staff of the Antibiotics Research Station of the Medical Research Council. I have felt it to be a privilege to lecture on these microbial products at Rutgers University, where some very important discoveries in the field of antibiotics have been made.

E. P. ABRAHAM

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1

THE BACITRACINS

The name bacitracin was given by Johnson, Anker, and Meleney,[1] in 1945, to an antibiotic produced by an organism of the *Bacillus subtilis* group. This organism had been isolated from tissue taken from a compound fracture of the tibia of a patient named Tracy. The new antibiotic was water-soluble, had a low toxicity to animals, and resembled penicillin in its range of antibacterial activity.

In 1944, Magarão, Arriagada, and Thales [2] reported that a strain of *B. subtilis* produced antibiotic material which lysed *Myco tuberculosis* and, in 1947, Arriagada brought specimens of material from Chile to Oxford. No agent that lysed the tubercle bacillus was subsequently encountered, but a strain of *Bacillus licheniformis* which was isolated from one of the specimens produced an antibiotic that was active against a number of Gram-positive bacteria and against the meningococcus and the gonococcus. The strain of *B. licheniformis* was labeled A5 and the active substance called ayfivin.

Early work on ayfivin showed that it probably consisted of a family of polypeptides. At that time reports on the properties of bacitracin were inconsistent and gave no clear indication of its chemical nature. However, in 1948, Barry, Gregory, and Craig [3] published the results of a preliminary analysis of the amino acids in purified bacitracin, and the similarity of the product to ayfivin at once became apparent.[4] Direct comparison of preparations of bacitracin and ayfivin subsequently revealed that the two products were virtually identical; the name ayfivin was therefore withdrawn.[5]

Production and Isolation of the Bacitracin Peptides

Bacitracin was first produced in shallow layers of soybean digest,[6] potato-dextrose broth,[7] or synthetic medium containing glucose, ammonium lactate, and inorganic salts. Hills, Belton, and Blatchley [8] made the interesting observation that *B. licheniformis* (strain A5) yielded bacitracin in one synthetic medium but an antibiotic with the characteristics of the peptide licheniformin in a similar medium with a lower carbon/nitrogen ratio. Later, bacitracin was produced in deep aerated cultures,[9] but material prepared from these fermentations was said to cause greater damage to the kidneys than earlier products.[10]

The first steps in the purification of bacitracin caused no great difficulty. The active material could be extracted from culture fluids at about *p*H 7 by butanol and returned to an aqueous solution at *p*H 3,[1] or adsorbed onto charcoal and eluted with a two-phase mixture of butanol and dilute acid,[4] and it could be precipitated from concentrates as a picrate. The best products obtained by such procedures contained a considerable proportion of antibiotic material, but it was soon evident that they were heterogeneous and consisted largely of a family of related polypeptides. A

number of methods have been applied to the resolution of these mixtures. Limited success was obtained by chromatography on charcoal and zone electrophoresis;[11] and, although the use of chromatography has been complicated by the size and instability of the peptide, it is possible that an efficient chromatographic process could now be found. So far, however, the most fruitful procedure for the separation of the bacitracins has been countercurrent distribution between solvents in the apparatus developed by Craig.

Separation of the Bacitracin Peptides by Countercurrent Distribution. Two different solvent systems were used in different laboratories for the separation of the bacitracin polypeptides. The first, which was chosen by Craig and his colleagues,[3,12,13] consisted of *sec*-butanol and 3% acetic acid. The second, employed by Newton and Abraham,[4,5,14] consisted of a mixture of *n*-butanol and amyl alcohol in equilibrium with phosphate buffer at *p*H 7.0. These two systems have different advantages and drawbacks. System 1 has a high capacity and provides conditions under which bacitracin is relatively stable, but its resolving power is low and the large numbers of transfers required for its effective exploitation can only be conveniently carried out in an automatic apparatus. System 2 has a much higher resolving power, but its capacity is lower and because of the limited stability of bacitracin at *p*H 7 special precautions, including the use of highly purified solvents, are required to prevent transformation of the peptides during the distribution.

Figure 1 shows the result of countercurrent distribution of crude bacitracin in system 2. The major active component of the mixture, whose ultraviolet absorption spectrum showed a maximum at 253 mμ, was called bacitracin A. The letters C, G, and F were assigned to components that were more soluble than A in the alcoholic phase; and B, D, and E were assigned to components that were less soluble.

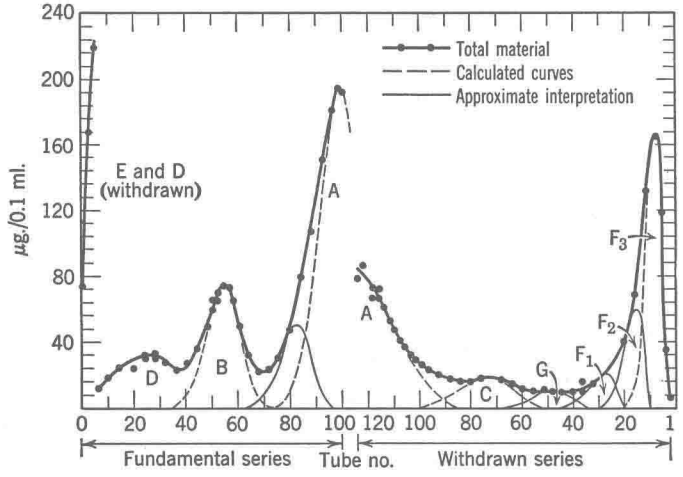

Fig. 1. 221-transfer distribution of crude bacitracin in amyl alcohol–*n*-butanol (4:1): 0.05 *M* potassium phosphate buffer, *p*H 7.0. From *Biochem. J.*, **53**, 597 (1953).

The ultraviolet absorption spectrum of bacitracin F showed a maximum at 288 mμ. Bacitracins B and C showed considerable antibacterial activity, though less than that of A, but the activities of the remaining components were relatively low. It was disappointing to find that bacitracins A, B, and C were not devoid of the nephrotoxicity which had limited the clinical use of cruder products,[15] but the subsequent discovery that bacitracin F was at least as toxic as bacitracin A showed the desirability of eliminating this inactive component from commercial material.[16]

It is a reasonable assumption that bacitracin A, as prepared by countercurrent distribution in either system 1 or 2, represents a single entity, but the homogeneity of bacitracins B and C is less certain. A peptide with the ultraviolet absorption spectrum of bacitracin F was shown by Craig,

Weisiger, Hausmann, and Harfenist [13] to be formed when bacitracin A was kept in a neutral or slightly alkaline solution, and it is probable that the bacitracin F complex (Fig. 1) represents the result of non-enzymic transformations which have occurred in the culture fluid, or during the preparation of the crude material. A different type of transformation may be responsible for the presence of bacitracins D and E. Unlike the other compounds, bacitracin E has apparently been obtained in a crystalline state.[13]

Bacitracin A, which accounts for most of the antibacterial activity of crude bacitracin, is the member of this family of peptides that has received the most detailed chemical study.

The Nature of Bacitracin A

If bacitracin A is compared with some of the proteins whose structures are now beginning to be unraveled, it appears to be a relatively small molecule. Nevertheless, the determination of the amino acid sequence in bacitracin A did not prove to be a simple task, and certain details of its structure still remain to be settled. A number of factors contributed to this situation, among them the arrangement of D-amino acids in the molecule and the inability to make use of the hydrolytic enzymes that have proved so helpful in work on substances such as insulin, hypertension, and ribonuclease.

Molecular Weight and Amino Acid Composition. Anker, Johnson, Goldberg, and Meleney [17] stated that bacitracin would diffuse through a nitrocellulose membrane which held back particles of molecular weight greater than 2000, and from the results of electrometric titration Newton and Abraham [18] concluded that the weight of the smallest unit which could represent bacitracin A was close to 1500. Craig, Hausmann, and Weisiger [19] studied the problem by a new

method for the determination of the molecular weight of peptides, depending on partial substitution, which had been devised by Battersby and Craig.[20] They separated the products formed when bacitracin A reacted with 1-fluoro-2,4-dinitrobenzene (FDNB) by countercurrent distribution in *sec*-butanol–3% acetic acid. From the optical density, at 350 mμ, of what appeared to be an N-monosubstituted dinitrophenyl (DNP) derivative, they deduced that the molecular weight of bacitracin A was about 1470. Since all these results were consistent, there could be little doubt about the approximate size of the molecule.

Analyses on starch columns,[3] on paper chromatograms,[18] and on Dowex 50 resin [21] of acid hydrolysates of bacitracin A gave a quantitative picture of the amino acid residues in the molecule. In one detail, however, concerning the number of isoleucine residues present, this picture was inaccurate, and the situation was only clarified when studies on the sequence of amino acids were almost complete. The possibility that the values obtained for some of the amino acid residues were low had been appreciated, since peptides containing isoleucine and phenylalanine were still present among the products from hydrolysis of bacitracin with 6 *N* HCl at 100° for 22 hours.[3] Nevertheless, the value of two residues, which was assigned to isoleucine, was lower than it should have been, partly because an amino acid which emerged from the column of ion exchange resin as a small peak immediately preceding that due to isoleucine was not at first identified. In 1954, Piez [22] pointed out that diastereoisomers of amino acids with two asymmetric centers could be separated on ion exchange resins and that the unidentified substance was probably alloisoleucine. The conclusion could then be drawn that this amino acid, which was present in hydrolysates of bacitracin A in half a molecu-

lar proportion, was formed, by racemization, from isoleucine.[23,24]

Early experiments with D-amino acid oxidase indicated that some of the amino acids in bacitracin had the D-configuration.[4] Craig et al.[21] separated the amino acids formed on hydrolysis of 3 grams of bacitracin by countercurrent distributions, involving over 2000 transfers, in a number of solvent systems. They were able to isolate the amino acids in crystalline form and, by determining their optical rotations, to show that phenylalanine, ornithine, and glutamic acid were D-isomers, whereas aspartic acid was racemic and the remaining substances were L-isomers.

The evidence now available indicates that the molecule of bacitracin A is composed of the twelve amino acid residues shown in Table 1. In addition, one molecule of

TABLE 1

Amino Acid Composition of Bacitracin A

Amino Acid	Number of Residues
L-Leucine	1
L-Isoleucine	3
D-Phenylalanine	1
L-Cysteine	1
L-Aspartic acid	1
D-Aspartic acid	1
D-Glutamic acid	1
L-Histidine	1
L-Lysine	1
D-Ornithine	1
"Amide" N	1

ammonia, which might well come from an amide group, is liberated by hydrolysis with hot acid or cold alkali.

Ionizable Groups and N-Terminal Residue. Electrometric titration of bacitracin A in aqueous solution showed that