Antibiological Polypeptides as Illustrated by Circulin[1]

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Introduction

In the summer of 1946 Murray and Tetrault (8) began a search for new antibiotics active against gram-negative organisms. This seemed appropriate because at that time no drug was available that would serve as specific remedy against such diseases as typhoid fever, paratyphoid fever, dysentery, cholera, certain kidney infections, etc.

Since several members of the genus *Bacillus* were known to produce peptide antibiotics that were relatively toxic, Murray (7) avoided picking aerobic spore-forming bacilli as potential antibiotic-producers. It was indeed fortunate for the development of circulin that, for a short while at least, he considered his soil isolate Q-19 to be a non-spore-forming organism; otherwise he probably would have discarded what turned out to be a promising if slightly unorthodox strain of *B. circulans*. In further studies on the antibiotic products of this organism Murray and Tetrault were joined by Koffler, Kaufmann, Quinn, Napoli, Perry, Reitz, and Dowling at the Purdue laboratories, by a group led by Peterson and Colingsworth at the Upjohn Company, by Nash at Pitman-Moore, and by workers at various medical institutions.

Biological properties

These studies have shown that *B. circulans* Q-19 produces several antibiotics (in this paper referred to as circulin fractions 1, 2, and 3) that appear to be chromatographically different (3, 11, 14). Circulin is both biologically and chemically similar to the five known polymyxins (A, B, C, D, and E), which are antibiotic polypeptides produced by various strains of *B. polymyxa* (1,2,3,5,9,10,11,13,14,15,16,18). Like them it is selectively active against many gram-negative bacteria such as members of the *Enterobacteriaceae*, to which several intestinal parasites and also the plant pathogen *Erwinia* belong (1,9,13,15,18). Interestingly enough *Proteus* and *Serratia* are the only genera of the *Enterobac-*

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[1] The antibiotic described in this review is not identical with the highly toxic product of *Bacillus krzemieniewski* M-14, which for a brief period of time was also known as circulin but which is now called polypeptin (4, 6).

The author is grateful to his coworkers at Purdue University and Dr. Harold Nash of the Pitman-Moore Company in Indianapolis for graciously making available unpublished data that were summarized in this review. Much information on the structure of circulin (fraction 1) was contained in a correspondence between Dr. R. G. Sheperd, of the American Cyanamid Company, Stamford, Connecticut, and Dr. D. H. Peterson, of the Upjohn Company, Kalamazoo, Michigan. The author is indebted to Dr. Peterson for enabling him to benefit from this exchange of letters.

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teriaceae that are strikingly resistant to circulin (9, 13, 18). Not only is circulin active *in vitro*, but it also was shown to protect white mice from infections caused by *Klebsiella pneumoniae*, *Salmonella typhosa*, and *Vibrio cholerae*, and to be useful in the control of human kidney infections caused by members of the genus *Pseudomonas* (1,9,13,15). This pronounced action against certain gram-negative bacteria together with the fact that such organisms develop resistance to circulin only with difficulty (10,18) make it a potentially useful therapeutic agent, especially against infections caused by pathogens that have become resistant to other drugs. However, circulin is fairly toxic to experimental animals (9,13,15,16), and at this stage should be used only in clinical trials.

Little is known of the mechanism by which circulin acts against susceptible cells. It appears to be bactericidal (10,18), and in many ways behaves like a cationic detergent. However, cationic detergents tend to be more active against gram-positive bacteria than they are against gram-negative organisms; the opposite is true for circulin. Whether the mode of action of circulin depends upon the physical disintegration of the cytoplasmic membrane, metabolite-antimetabolite relationships, etc., still remains to be demonstrated.

Preparation

Circulin has been produced by growing *B. circulans* Q-19 either in shake flasks (9,12,15) or in 100-gallon pilot fermenters (12) in relatively simple media (9,12,15). As in other submerged fermentations the composition of the media, the pH, the degree of aeration and agitation, etc., are critical (9,12). Under optimum conditions the organism gives yields equivalent to approximately 1 mg. of pure circulin fraction 1 per ml.; however, about 60% of this is lost during recovery. In the pilot plant extraction procedures have been used with sulfonated alkyl aryl compounds (Ultrawet and Onyx Aliphatic Ester Sulfate) as specific precipitants. The circulin complexes formed on addition of these compounds dissolve when treated with acidified aqueous n-butanol or acetone, and circulin salts (for example sulfates when sulfuric acid is used to acidify the organic solvents) precipitate and are recovered (12). In the laboratory circulin fractions can be prepared by a method that in brief includes adsorption of the antibiotics from the clarified culture filtrate on activated carbon (Darco G-60) and elution with acidified aqueous tertiary butanol solutions (9,14). Further separation of the fractions can be achieved with a Celite 545, n-butanol, citrate-HCl system (3,11). The determination of purity in the case of polypeptides is very difficult. Usually one continues purification until the product shows constancy of composition, solubility, and biological activity; homogeneity of chemical and physical properties, etc. Unfortunately many organisms produce a series of compounds structurally so related that their separation becomes practically impossible. Even worse, frequently work is done on what appears to be a pure material and then turns out to be a mixture of substances. The history of circulin illustrates these points.

Peterson and Reineke (14) did some impressive chemical work on a compound (circulin fraction 1) that they obtained after repeated chromatography over Darco G-60 and that they considered to be essentially homogenous. One of the criteria used to establish this was that fraction 1 could not be resolved into more than one component by paper chromatography, with a developing solution that consisted of 25% water, 50% n-butanol, and 25% glacial acetic acid. By the time they had finished their chemical studies they had used up all of fraction 1. When Nash (11) later tried to repeat isolation of fraction 1 from less pure batches of circulin he found that he could isolate at least two components (the faster moving fraction 2 and the slower moving fraction 3) using chromatography with his Celite 545, n-butanol, citrate-HCl system. It seems significant that Peterson and Reineke's solvent system usually is not capable of accomplishing the separation of fractions 2 and 3 on paper chromatograms, while a system composed of the following does allow separation: 49.5% water, 49.5% n-butanol, and 1.0% glacial acetic acid (3,11). This observation raised the question of whether Peterson and Reineke had dealt with a single antibiotic (probably identical to fraction 2) or with a mitxure of fractions 2 and 3. Since Peterson and Reineke's fraction 1 was exhausted by analysis this cannot be answered with certainty. Recent data, however, make the first possibility more likely. Using Peterson and Reineke's method of purifying circulin, Peterson (3) was able to resolve impure circulin into two or more components, one of which behaved chromatographically the same as did his previous fraction 1 or Nash's fraction 2. This preparation appeared homogenous on paper chromatograms even when a solution of 49.5% water, 49.5% n-butanol, and 1% glacial acetic acid was used. Apparently Peterson and Reineke's original developing system (14), though incapable of separating fraction 1(=2) from fraction 3 on filter paper, did permit resolution when Darco G-60 was used as supporting material. In connection with the purity of the circulins it should be remembered that all the biological studies mentioned previously were made with preparations that contained two or more components.

Chemical properties

Circulin fraction 1 is a basic polypeptide that can be converted easily into its picrate, helianthate, reineckate, hydrochloride, sulfate, etc. The sulfate and hydrochloride of fraction 1 are highly soluble in water, and relatively insoluble in water-immiscible solvents. Fraction 1 was reported to have the same qualitative composition as polymyxins A and E, and all three antibiotics contain L-threonine, D-leucine, L-α, γ-diaminobutyric acid (DABA), and a fatty acid with the properties of 6-methyloctanoic acid (2,5,14,17). In fraction 1 these constituents are thought to occur in a ratio of 1:1:5:1, and the amino acids have been assumed to be arranged in a cycle (14). Peterson and Reineke (14) based their claim for the existence of such an arrangement on 1) the amino acid composition; 2) the fact that approximately one-half of the amino nitrogen of circulin is uncombined (the amino nitrogen before hydrolysis was 7.5 per cent after hydrolysis 15.8 per cent); 3) the

absence of free carboxyl groups as shown by titration curves and a negative ninhydrin-CO_2 (Van Slyke) test; and 4) evidence that the amino groups of DABA were the only free amino groups in circulin. The fourth line of evidence is subject to some question and is being reinvestigated (3). It was based on the observation that the 2,4-dinitrophenyl (DNP) derivative of circulin when hydrolysed with hydrochloric acid apparently yielded no other products than DABA, α-amino-γ-(2,4-dinitroanilino)-butyric acid, threonine, and leucine, as observed by paper chromatography. However, since DNP derivatives of monoamino acids do not react with ninhydrin, which was used to indicate the position of the various components on the chromotogram, and are visible only by their yellow color, small quantities of such derivatives may have escaped detection. Moreover, the fact that not all of the DABA was converted to its DNP derivative raises the question of whether this was true because the dinitrophenylation reaction was not carried to completion or because some DABA is combined in the intact molecule. Inasmuch as the dinitrophenylation was performed in the absence of alcohol, a condition under which the reaction is thought not to go to completion (3), the first alternative seems more likely. DABA is not formed from its DNP derivative by acid hydrolysis (3), and this possibility can therefore be excluded as an explanation for the occurrence of free DABA on the chromatogram. Although it is plausible that circulin has a cyclic structure, as presented in the generalized formula given in Figure 1, the contentions that all of its free amino groups are furnished by DABA

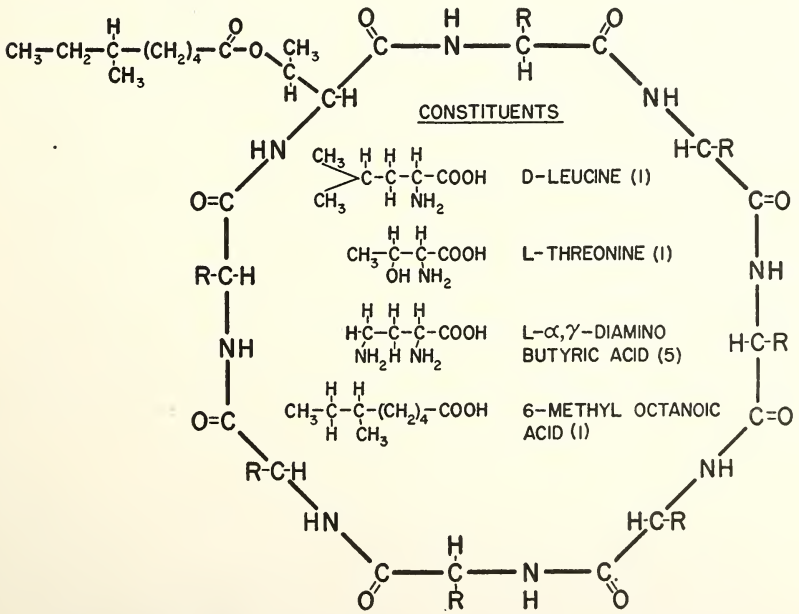

Fig. 1. Generalized hypothetical structure of circulin (fraction 1).

and that all DABA side chains are unsubstituted still remain to be verified. Proof of these and of a cyclic structure is prerequisite also to an interpretation of the behavior of circulin in the presence of lipase. Circulin, and polymyxins B and E are inactivated by lipase, while polymyxins A and D are not (3,14); polymyxin C has not yet been tested. The fact that circulin is inactivated by lipase has been regarded as suggestive evidence that 6-methyloctanoic acid is joined to the polypeptide through threonine by an O-acyl linkage (14), as is tentatively visualized in Figure 1. This needs to be so only if circulin is a cyclic polypeptide, all of its free amino groups are furnished by DABA, and all DABA side chains are unsubstituted, conditions that have not yet been met fully. One will also have to demonstrate that the lipase preparation used is able to hydrolyze O-acyl but not N-acyl linkages. Isolation of the fatty acid and the intact polypeptide after inactivation of circulin by lipase, and demonstration that a hydroxyl group rather than amino group becomes liberated during inactivation are necessary before any view on the manner in which the fatty acid is attached to the rest of the molecule can be accepted (3). If all these requirements are fulfilled it will be interesting to see whether the polymyxins and circulins can be divided into those in which the fatty acid is attached through an N-acyl linkage and those in which an O-acyl linkage exists.

Since it was claimed that fraction 1, and polymyxins A and E have qualitatively the same composition, it became necessary to determine whether these three were different antibiotics. That fraction 1 and polymyxin A are not identical became apparent from their different resistance to inactivation by lipase (14). However, polymyxin E was not available for comparative work at that time, and the possibility could not be ruled out that fraction 1 and polymyxin E were the same. Since fraction 1 was not available for further comparison, fractions 2 and 3 were eventually studied chromotographically, and found to be different from polymyxin E (3). However, another uncertainty still clouds the issue. Nash (11) recently has obtained preliminary evidence that both fractions 2 and 3 contain another amino acid, probably isoleucine. Although this evidence remains to be examined more thoroughly, it suggests that the circulins 1 and 2, contrary to previous notions, may differ from polymyxin E in qualitative composition. Confusing as the situation may be, it is rather typical of the manner in which our knowledge on the nature of antibacterial polypeptides evolves. This, however, is no consolation and it is somewhat discouraging to reflect upon the prospect that a few years from now circulin may be found to consist not of two or three but of several entities.

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