Bacteriolytic Enzymes from Streptomyces

A review

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ABSTRACT A study of the bacteriolytic properties of streptomyces has progressively uncovered the production by these microorganisms of a large number of different enzymes acting upon various bacterial constituents, especially on some of them located in the cell wall. Although the mechanism of the bacteriolysis is far from being completely elucidated at present, it can, however, be stated that, in two instances at least, it can be regarded as an osmotic explosion following upon the destruction of the structure responsible for the rigidity of the cell wall.

Leaving aside the often quoted but rather incidental observation of Gasperini (1890), it may be stated that the ability of *Streptomyces* spp. to lyse the bacterial cells of different species, either previously killed or alive, was independently discovered by Lieske (1921) and by Gratia and Dath (1924). The latter demonstrated that the lytic activity could be recovered in the cell-free filtrate from a suitable actinomycete culture. Similar reports were published during the following years (Rosenthal, 1925; Dath, 1927; Borodulina, 1935; Gratia and Alexander, 1931) and, later on, by analogy with the word "penicillin," as originally introduced by Fleming (1929), we proposed to designate such bacteriolytic cell-free fluids as actinomycetin (Welsch, 1937 c).

The actinomycetin type of lytic activity, which is quite widespread among Actinomycetes (Welsch, 1942, 1947 c; Bergamini, Ghuysen, and Welsch, 1954), can be described as follows:

(a) The active actinomycete, when planted in a suspension of properly killed bacteria belonging to any one of a large number of species tested, will grow and dissolve the bacterial bodies.

(b) The same result obtains when the actinomycete is planted in a suspension of living bacteria belonging to a wide variety of Gram-positive species.

(c) The cell-free fluid, obtained after centrifugation or filtration of a culture of the actinomycete, in short, actinomycetin, shows the bacteriolytic activities described under (a) and (b), although its effects upon living bacteria are often somewhat more restricted and may be rather irregular.

A microorganism, which we isolated in 1936 (Welsch, 1936) and which...
was described later on as *Streptomyces albus* G (Waksman, Horning, Welsch, and Woodruff, 1942; Welsch, 1947 c), maintained throughout the years in a suitably active state by selection procedures, was used in our laboratory as the typical actinomycetin producer. In many instances, however, results obtained with the aforementioned organism were extended to a large number of other strains, mostly unidentified, maintained as a reference culture collection.

1. **First Hypothesis: A Single Lytic Enzyme**

Early studies, bearing on the physical and chemical properties of the agent of actinomycetin G responsible for its lytic action on killed bacterial bodies, strongly suggested that it was a protein, while the kinetics of bacteriolysis indicated that it very likely acted as an enzyme (Welsch, 1937 b, 1947 c; Welsch and Elford, 1937). Its purification was therefore attempted, using the methods devised by Northrop (1939) and his collaborators for the isolation of crystalline enzymes. Unfortunately, the exceedingly low concentration of active protein in actinomycetin proved to be a very serious handicap to its purification by the salting-out technique. However, the activity of the still very crude but concentrated preparations thus obtained, when compared with that of crystalline trypsin and other highly purified proteases against various substrates, showed a specificity for killed bacterial bodies which warranted postulating the presence of a special kind of enzyme, distinct from the ordinary proteases (Welsch, 1941). The same conclusion was also reached, later on, by Tai and van Heyningen (1951), Born (1952), and Klinge (1955) working with different actinomycetes.

The substrate mainly used in our study of the lysis of dead bacteria was heat-killed *Escherichia coli* and therefore the responsible enzyme was called the colilytic agent, or, later on, to stress its individuality as well as its ability to act upon various bacteria, actinozyme (Welsch, 1947 a).

The lysis of living bacteria by actinomycetin was mainly studied with *Staphylococcus pyogenes* as a substrate, although *Bacillus megaterium* and a few other Gram-positive microorganisms were also used. Since any sample of actinomycetin was generally found able to dissolve several kinds of living bacteria, it was first assumed that these activities were due to a single so called staphylolytic agent. Similarly, the staphylolytic type of activity never having been found in the absence of the colilytic type, we proposed a unitarian hypothesis (Welsch, 1947 a) according to which actinomycetin contained only a single bacteriolytic agent, namely actinozyme. Then, in order to explain its action upon living Gram-positive bacteria (Welsch, 1937 a; Swertz, 1949; Smoliar, 1950), which is clearly a manifestation of antibiosis (Welsch, 1947 b), we were led to assume that a bactericidal agent was present.
in actinomycetin, which first killed the microorganisms thus making them sensitive to the lytic effect of actinozyme. The kinetics of staphylolysis (Welsch, 1947 c) could easily be interpreted along those lines and, furthermore, a material of a lipoid nature, specifically bactericidal for Gram-positive bacteria, was extracted from actinomycetin, although it was also found, albeit in much smaller amounts, in the sterile culture medium (Welsch, 1941).

2. Second Hypothesis: A Single Lytic System of Several Enzymes

Working with another strain of *Streptomyces*, Jones, Swallow, and Webb (1948) and Muggleton and Webb (1952) explained its lytic action upon Gram-positive bacteria as a complex sequence of reactions. The microorganisms, killed by a hypothetical antibiotic-like substance, were supposed to be next converted into Gram-negative cytoskeletons through the action of a specific ribonuclease. These, in turn, were dissolved thanks to the effect of two different proteases which together were shown to be able to lyse heat-killed Gram-negative cells.

Although the presence in the active fluid of the several enzymes supposed to be involved was clearly demonstrated, the description of lysis as a multiple step mechanism can only be regarded as a plausible, but hypothetical, reconstruction. Its applicability is furthermore restricted, since we had already shown that the bactericidal lipoids of actinomycetin G play no necessary part in its various lytic activities, these being left unimpaired after their removal (Welsch, 1947 c). It must be added, however, that a number of staphylolytic streptomycetes exert a bactericidal action upon staphylococci which is ascribable to an agent more diffusible than the lytic one (Smoliar, 1949). Further, we were unable to detect ribonuclease in many samples of actively lytic actinomycetin G, by either direct chemical or indirect cytochemical tests (Welsch, 1948). Finally, in our opinion, the idea that conversion of Gram-positive bacteria to Gram-negative ones by a ribonuclease is a prerequisite to bacteriolysis is left open to discussion (Welsch and Salmon, 1950; Welsch and Delcambe, 1951; Welsch, 1957 b).

3. Third Hypothesis: A Multiplicity of Specific Lytic Enzymes

The idea that actinozyme was the only truly lytic enzyme in actinomycetin had to be discarded when Ghuysen’s observations conclusively showed that the colilytic and staphylolytic types of activity could be ascribed to distinct agents. In fact, their biosynthesis is differently influenced by the composition of the medium and cultural conditions. The physicochemical factors necessary for their respective optimal activity are different. They can be separated one from the other by several procedures of adsorption and elution and these
techniques have shown, not only that staphylolysis by actinomycetin is still possible after removal of the colilysic agent or actinoyzme, but also that the latter is distinct from several other enzymes present in the crude fluid (Ghuy-
sen, 1952; Ghuysest and Léger, 1954; Ghuyseen, Léger, and Dierickx, 1959),
including a casease, a keratinase, a skin-depilating factor, which is very
likely responsible for the separation of the epidermal layer of skin fragments
from the dermis (Castermans, 1956), and a virus-inactivating agent (Mal-
chair, 1958). It should be added that some actinomycetin samples are also
a source of chitinase (Jeniaux, 1950) and of mucases acting on salivary

gland mucin (Bergamini, 1954).

At the same time, evidence for the existence of a streptolytic enzyme
distinct from the colilysic agent was given by McCarty (1952), working
with one of Maxted's strains of *Streptomyces albus* known to dissolve the cells
of group A streptococci with liberation of their group-specific C carbohydrate (Maxted, 1948). That mycolysates contain a number of the bacterial
antigens was in fact known since the earlier observations of Gratia and Dath
(1924) and such lysates had already been used as vaccines (Gratia, 1930).

Release of various bacterial antigens after lysis was studied in our labora-
tory by Castermans (1950) and by Salmon (1957).

The purified streptolytic fraction obtained by McCarty, which had only
weak proteolytic properties, induced a rapid conversion of Gram-positive
bacteria to Gram-negative ones and morphological alterations of the cells
as well as a slowly increasing, but incomplete, clearing of the streptococcal
suspensions. The addition of another fraction capable of digesting casein and
of lysing heat-killed suspensions of *E. coli*, but having no action by itself on
streptococci, resulted in a quicker and more complete lysis. However, the
same effect was obtained by the addition of other proteolytic enzymes such
as trypsin.

The streptolytic fraction contained only traces of ribonuclease activity and
its potency was not increased by the addition of crystalline pancreatic ribo-

nuclease. A lysozyme-like enzyme, detected in the crude preparation, was
absent from the streptolytic fraction. It was potent in reducing the viscosity
of the specific substrate isolated according to Meyer and Hahnel (1946), but
had only a weak activity on suspensions of *Micrococcus lysodeikticus*. Let it be
recalled that the bacteriolytic properties of actinomycetes had been attributed,
without much experimental evidence, to a lysozyme-like enzyme by Kras-
silnikov and Koreniako (1939) and by Kriss (1940), but that we rejected that
explanation, in the case of actinomycetin G, on account of its very weak
action on *M. lysodeikticus*, then generally considered as the most sensitive
substrate (Welsch, 1942, 1947 c).

McCarty was therefore entitled to conclude that streptolysis was due to,
or at least initiated by, a specific non-proteolytic enzyme, other than ribo-
nuclease or lysozyme, complete clearing of the streptococcal suspensions requiring, however, the participation of a non-specific protease. He next demonstrated that the streptolytic enzyme dissolved the isolated and purified protease-resistant cell walls of streptococci, acting very likely on their carbohydrate moiety. Thus was introduced the notion that bacteriolysis by streptomycetes might well be the consequence of a specific enzymic destruction of the bacterial cell wall.

McCarty's work prompted us to investigate whether his streptolytic and our staphylolytic enzyme were identical. This led to the discovery that lysis of staphylococci and lysis of streptococci were two different properties of actinomycetin G (Ghuysen and Welsch, 1952; Welsch and Ghuysen, 1953), and, further, that the two types of activity were not perfectly correlated in a series of streptomycete cultures (Welsch, 1954; Bergamini, Ghuysen, and Welsch, 1954). Soon afterward, Ghuysen (1954) demonstrated the presence, in actinomycetin G, of two more distinct lytic enzymes, both acting on pneumococci. The notion of a multiplicity of highly specific bacteriolytic enzymes, also strengthened by Casterman's observation on the lysis of Gram-negative rods by actinomycetin at different temperatures (Castermans, 1955), was therefore firmly established (Welsch, Ghuysen, and Castermans, 1956; Welsch and Ghuysen, 1956; Ghuysen, 1957).

4. The Staphylolytic System of Actinomycetin G

The realization that actinomycetin contained several lytic agents, each endowed with its specific range of activity, made the task of elucidating the mechanism of bacteriolysis by streptomycetes even more formidable than had at first appeared.

At the present time, only one of these agents, namely the so-called staphylocytic agent, has been studied in some detail and shown to be a very complex system.

With the use of ion exchangers, Ghuysen (1957) isolated from crude concentrated actinomycetin a fraction F 1 which contained a basic protein, homogeneous enzymically as well as from the results of several physico-chemical tests. Fraction F 1, without any action on E. coli, even after heat-killing, induced a more or less rapid clearing of the suspensions of Staphylococcus pyogenes, Micrococcus lysodeikticus, Bacillus subtilis, B. megaterium, and B. brevis. It also lysed streptococci and pneumococci, but was different from the specifically streptolytic and pneumolytic agents mentioned previously.

Ghuysen also observed that another fraction, F 2, still very crude and containing, among other enzymes, several proteases, actinonezyme, and the pneumolytic agents, having by itself a very weak staphylocytic action, was nevertheless able to potentiate the lysis of staphylococci by F 1. This effect
was ascribable to a so called complementary staphylolytic agent different from the aforementioned enzymes.

Salton and Ghuysen (1957) showed that F 1 digested the purified cell walls from *Staphylococcus pyogenes*, *Sporosarcina ureae*, *Bacillus subtilis*, *B. megar-terium*, *B. cereus*, *Clostridium welchii*, *Micrococcus lysodeikticus*, weakly those of *Sarcina lutea*, and not at all those of *Escherichia coli*. On the contrary, F 2 had very little action on the six first substrates but potentiated their digestion by F 1. By itself, F 2 digested the cell walls of *M. lysodeikticus* and *S. lutea*.

Solubilization of cell walls by F 1, F 2, or F 1 and F 2 together, was accompanied by the release of non-dialyzable and dialyzable products. Among the latter alanine and/or glycine, together with small unidentified peptides, was regularly detected and it was first assumed that the active enzymes were peptidases. However, further research, using more sensitive analytical procedures (Salton, 1959), showed that F 1 released from the cell walls of *M. lysodeikticus* hexosamine compounds of various complexity. The structure of several of these compounds was established and it was demonstrated that F 1 contains an enzyme which, like egg white lysozyme, possesses the activity of a β-(1-4)-N-acetylhexosaminidase (Ghuysen and Salton, 1960; Ghuysen, 1960a, 1961a; Salton and Ghuysen, 1959).

These observations indicated that fraction F 1 was not so pure as was thought initially. In fact, Ghuysen has shown that, in addition to the hexosaminidase, which has now been highly purified by zone electrophoresis in a gradient of sucrose (Dierickx and Ghuysen, 1961), it contained an enzyme able to release the teichoic acids from the muropeptide to which they are bound in the cell wall of *B. megar-terium* (Ghuysen, 1961b).

It should be stressed at this point that hexosaminidase F 1 and egg white lysozyme are not identical. The former is a protein a little less basic than the latter. Both enzymes display generally the same activities qualitatively but not quantitatively. For example, they digest equally well the purified cell walls of *M. lysodeikticus*, but the former is far less active than the latter on whole cell suspensions of this microorganism. They differ sharply in at least one respect: the central β-(1-4) linkage of the tetrascaride: O-β-N-acetylglucosaminyl-(1-6)-O-β-N-acetylmuraminyl-(1-4)-O-β-N-acetylglucosaminyl-(1-6)-β-N-acetylmuramic acid, which is easily split by lysozyme, is entirely resistant to the purified hexosaminidase F 1. However, an enzyme capable of splitting that linkage could be detected in some samples of crude actinomycecin but was not recovered after fractionation by zone electrophoresis. This procedure, on the other hand, permitted the isolation of a chitobiase which, apparently, plays no role in the digestion of cell walls from *M. lysodeikticus* (Dierickx and Ghuysen, 1961).

Preparation F 2 from actinomycecin was in turn separated into two fractions F 2A and F 2B, the former containing the complementary staphylolytic
agent, actinozyme, and several proteases. The latter, containing a casease, had no potentiating effect on the staphylolysis by F 1, but favorably influenced the lysis of staphylococci by the already synergic association of F 1 and F 2A. By itself, fraction F 2B lysed slowly the suspensions of \textit{M. lysodeikticus,} but not those of \textit{Staphylococcus pyogenes.} It digested the cell walls of \textit{M. lysodeikticus} and weakly those of \textit{S. pyogenes} and \textit{B. megaterium} (Ghuysen, 1960 a). These activities were attributed, at least in part, to an enzyme which was shown to split the amidic acetylmuraminyl-alanine linkage (Ghuysen, 1961 a).

5. Mechanism of Bacteriolysis

In the course of these studies, it was oftentimes observed that the physico-chemical conditions most favorable for the digestion of cell wall preparations were identical with those that were optimal for bacteriolysis. It was therefore concluded that at least some of the bacteriolytic activities of actinomycetin G, and of streptomycetes in general, like that of lysozyme (Salton, 1952), were the consequence of an enzymic destruction of some important constituent of the bacterial cell wall. This view was substantiated by the fact, reported by Salton, that streptomycetes dissolving bacterial cell walls can be easily isolated from natural substrates by plating on agar containing cell walls (Salton, 1955). Following Weibull’s observations (Weibull, 1933), it is now well established that bacteriolysis can be the result of the osmotic explosion of a wall-less protoplast or of a wall-deficient spheroplast (Welsch, 1958), but that those fragile structures can be preserved in a suitable medium, such as a solution of sucrose of high enough concentration. When bacteria, submitted in such a medium to an otherwise lytic agent, are converted into protoplasts or spheroplasts, it means that lysis, in the particular instance, can be brought about solely by a cell wall-destroying agent. But, unless a perfectly pure substance is used, the actual participation of one or several other agents cannot be excluded.

Thus, Gooder and Maxted (1958), and later Slade and Slamp (1960), demonstrated that McCarty’s carboxydrase converted streptococci into spheroplasts. Ghuysen (unpublished data) obtained protoplasts of \textit{B. megaterium} KM through the action of F 1 and made the interesting observation that the stability of these structures in concentrated sucrose was different in accordance with the nature of the culture medium used for bacterial growth. Similarly we studied the action of crude actinomycetin and of its fractions F 1, F 2A, and F 2B upon suspensions in 20 per cent sucrose of \textit{Bacillus megaterium} 890, \textit{B. subtilis, E. coli,} and several streptomycetes (Welsch, 1959, 1960). Our observations showed that F 1 converted all these organisms into spheroplasts, provided, in the case of \textit{E. coli,} that the bacteria had pre-
viously been treated by freezing and thawing or lyophilization. Crude actinomycin also produced spheroplasts, more slowly as could be expected, but they were very unstable even when the concentration of sucrose was raised. In fact, the crude preparation exerts a destructive action on spheroplasts obtained by various means, such as glycine or penicillin (Welsch, 1957a; Welsch and Osterrieth, 1958). Fractions F 2A and F 2B showed much less lytic activity than F 1 and, in sucrose, produced only few or no spheroplasts: the mechanism of their action therefore remains doubtful.

CONCLUSIONS

The study of the bacteriolytic properties of streptomycetes started with the use of whole bacterial cells as a substrate. These were next replaced by purified cell walls and, finally, by chemically defined compounds, in some cases released from the walls themselves. Thus, a number of specific lytic agents and more or less well defined enzymes have been uncovered which have proved to be useful tools to study the structure of the bacterial cell wall.

With respect to the mechanism of bacteriolysis by streptomycetes, a great deal remains to be done since a correct interpretation is impossible unless pure preparations of each of the enzymes involved are available. At the present time, it may be safely stated that, in two instances, lysis can be regarded as the result of a degradation of a wall constituent, through the action of a single enzyme. Such is the case for streptolysis by McCarty's carbohydrase. Such is also the case for the lysis of several Gram-positive organisms and properly treated E. coli by hexosaminidase F 1 from actinomycetin G. The wide spectrum of activity of this enzyme, akin to but different from egg white lysozyme, is easily understood since it acts upon the basic structure of the cell wall supposed to be common to all bacteria (Work, 1957; Ghuysen, 1960b).

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