Production and Purification of Bacilysin

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1. Bacilysin, a hydrophilic substance formed by certain aerobic spore-forming bacteria that causes lysis in cultures of growing staphylococci, has been produced in aerated cultures of a strain of *Bacillus subtilis* (A14). A chemically defined medium was used, which contained glucose, Czapek-Dox salts and ferric iron. Production of bacilysin occurred, after a lag, while the culture was still undergoing rapid growth. 2. Bacilysin was adsorbed from the culture medium on Zeo-Karb 225 (SR 5) (H⁺ form) and eluted with aqueous pyridine. The crude material was purified by chromatography in pyridine-acetate buffers on columns of Dowex 50 (X2) and Dowex 50 (X8) respectively and by chromatography in aq. 70% (v/v) propan-2-ol on Sephadex G-25. 3. Purified bacilysin behaved as a single ninhydrin-positive substance when subjected to chromatography on paper in butan-1-ol-acetic acid-water and to electrophoresis on paper at pH 4.5 or pH 1.8. At pH 4.5 the substance behaved as though it had no net charge and at pH 1.8 it migrated towards the cathode.

The name bacilysin was first given to an antibiotic produced by a strain of *Bacillus subtilis* (N.C.T.C. 7197) that had been isolated from the soil at Oxford (K. Gilliver, E. P. Abraham & H. W. Florey, unpublished work quoted by Florey *et al.* 1949). This substance caused rapid lysis in cultures of *Staphylococcus aureus* (N.C.T.C. 6571) growing in a liquid medium, but a small proportion of the cells survived and soon multiplied to yield a highly resistant strain. On plates of nutrient agar seeded with *S. aureus* the substance produced clear zones in which were scattered isolated colonies of resistant organisms (Abraham, Callow & Gilliver, 1946). Bacilysin was subsequently found to be produced by several other strains of *B. subtilis*, including a strain named A14, and also by some strains of *B. pumilus* (Gilliver, 1949; Newton, 1949). It differed from other antibiotics from *B. subtilis* whose properties were defined clearly enough for a significant comparison to be made; but whether it is related to bacillin (Foster & Woodruff, 1946; Woodruff & Foster, 1946; Walton & Riches, 1962) remains to be ascertained.

Bacilysin appeared in the culture fluid when *B. subtilis* was grown in shallow stationary layers of potato-dextrose broth (K. Gilliver, E. P. Abraham & H. W. Florey, unpublished work quoted by Florey *et al.* 1949). Subsequently, it was produced in deep aerated cultures with a medium consisting of potato-dextrose broth, or of Czapek-Dox salts, glucose and corn-steep liquor (Newton, 1949). Preliminary studies showed that it was extremely

hydrophilic and that it was probably a peptide (K. Gilliver, E. P. Abraham & H. W. Florey, unpublished work quoted by Florey *et al.* 1949; Newton, 1949).

The present paper describes the production of bacilysin by *B. subtilis* A14 (N.C.I.B. 9593) in a chemically defined medium and its isolation in a nearly pure state.

METHODS

Assay of antibacterial activity. Bacilysin was assayed by the cylinder-plate method (Heatley, 1944) with *S. aureus* (N.C.T.C. 6571) as the test organism and cephalosporin C as a standard. A solution of bacilysin that gave a zone of inhibition with the same diameter as that given by 1 mg. of cephalosporin C (sodium salt)/ml. was arbitrarily assigned an activity of 10 units/ml. Crude bacilysin gave zones with diffuse edges. The purified antibiotic gave clear zones that had sharp edges and often contained colonies of resistant organisms.

Maintenance of B. subtilis A14. The freeze-dried organism was grown overnight in Oxoid broth at 37° and the resulting culture used to inoculate a similar culture in the same medium. The second culture was streaked on to slopes (1 oz.) of Oxoid nutrient agar and the latter were incubated at 37° overnight. These slopes were kept at 2° and transfers to fresh slopes made at monthly intervals.

Inoculum for cultures used to produce bacilysin. A transfer from a slope stored at 2° was made to a fresh slope and the latter incubated at 37° overnight. Single loopfuls from this slope were used to inoculate media (10 ml.) in inverted T-tubes (Kay & Fildes, 1950). For production on a larger scale the organisms from one slope were washed into 250 ml. of medium contained in a conical flask (1 l.) having four vertical indentations (1 cm. x 10 cm.). The flask was held at an angle of 45° and rotated about its vertical axis at

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200 rev./min. for 18 hr. at 35°. The resulting culture was then used as an inoculum.

Czapek-Dox salts-corn-steep liquor medium. This was similar to that used by Newton (1949), but contained half the normal concentration of Czapek-Dox salts and 1% (w/v) of glucose. The yield of bacilysin decreased when the amount of corn-steep liquor was increased above 0.5% (v/v), or the amount of glucose above 2% (w/v).

Chemically defined medium. For each litre of chemically defined medium ammonium acetate (3.43 g.), KH_2PO_4 (1.0 g.), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g.), KCl (0.5 g.), FeCl_3 (0.3 g.) and an 'oligodynamic' solution (Pollock & Kramer, 1958) (1 ml.) were dissolved in 980 ml. of tap water and the pH of the solution was adjusted to 7.0-7.2 with 5N-NaOH (about 1 ml.). The solution was sterilized by autoclaving and a solution (20 ml.) of glucose (10 g.) in water, which had been autoclaved separately, was added. The medium contained a precipitate, which was not removed.

Estimation of cell growth. This was estimated from measurements of extinction (after dilution of the culture if necessary) in a test tube ($\frac{5}{16}$ in. diam.) with a Spekker absorptiometer (Adam Hilger Ltd.) and a neutral-grey filter (H508).

Antibiotic production (1) In inverted T-tubes. Each tube (capacity 40 ml.) contained 10 ml. of culture and was rocked (50 times/min.) in a water bath at 34°.

(2) In 5l. fermenters. The fermenter used was model F5-307 from the New Brunswick Scientific Co. Inc. (New Brunswick, N.J., U.S.A.). Each of the three vessels contained 4l. of medium and the inoculum for each vessel consisted of 250 or 500 ml. of a culture grown in the same medium in indented conical flasks. A sterile 50% (v/v) mixture of Antifoam A (from Hopkin and Williams Ltd., Chadwell Heath, Essex) in Shell Risella Oil (1 ml.) was added to each vessel after autoclaving and additional antifoam was added automatically as required during the growth of the cultures. Cultures were normally grown with an aeration rate of 1 vol. of air/vol. of medium/min. and with stirring at 700 rev./min. Samples (about 15 ml.) were withdrawn at 1 hr. or 2 hr. intervals for test.

(3) In 100l. fermenters. Bacilysin was kindly produced in 100l. fermenters by members of the staff of the Microbiological Research Establishment, Porton, Wilts. Cultures were grown in the chemically defined medium used at Oxford and the course of fermentation was similar to that in the New Brunswick fermenter.

Paper chromatography and electrophoresis. Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.). Electrophoresis on paper was carried out for 45 min. at 60 v/cm., with an apparatus similar to that of Katz, Dreyer & Anfinsen (1959), in a mixture of 20% (v/v) acetic acid and 2% (v/v) formic acid (pH 1.8), or in pyridine-acetate buffer (66 mM with respect to acetate), pH 4.5.

The position of bacilysin on the papers was found by bioautography. The air-dried paper was placed in contact with a nutrient agar plate seeded with *S. aureus* (N.C.T.C. 6571) for 15 min. The paper was then peeled off and the plate incubated at 37° overnight.

Ninhydrin-positive substances were detected by spraying the paper with a solution containing ninhydrin and 2,4,6-collidine (each 0.16%, w/v) in butan-1-ol (Woiwod, 1949). Sulphur-containing compounds were tested for with the iodine-azide reagent of Sjöquist (1953). Substances con-

taining tyrosyl or histidyl residues were detected by spraying with a solution of diazotized sulphanilic acid prepared as follows: to 1 vol. of a solution containing sulphanilic acid (4.5 g.) and 12N-HCl (5.0 ml.) in 490 ml. of water at 0° was added 0.1 vol. of a solution of NaNO_2 (4.5%, w/v) and, after 2 min., 1 vol. of Na_2CO_3 solution (10%, w/v). This reagent was used within 10 min. of preparation.

Chromatography on ion-exchange resins. Dowex 50 (X2) and Dowex 50 (X8) (both 200-400 mesh) were used in columns 135 cm. $\times$ 1.8 cm. diam. and 140 cm. $\times$ 0.9 cm. diam. respectively. The columns were equilibrated with pyridine-acetate buffers, pH 4.6 (0.5N with respect to pyridine) and pH 5.0 (0.2N with respect to pyridine) respectively, both the acetic acid and pyridine being distilled (the latter from NaOH) before use. The same buffers were used for elution. Constant flow rates were maintained through the column with an LKB 4500A Miniflow micro-pump from LKB-Produkter (Stockholm, Sweden). Ninhydrin determinations were carried out on samples (25-100 μ l.) from alternate fractions, 1.0 ml. of a mixture (1:1, v/v) of the ninhydrin reagent of Moore & Stein (1948) and 4M-sodium acetate (Moore & Stein, 1954) being used with each fraction. After the addition of 50% (v/v) ethanol (10 ml.) colour densities were measured in an EEL photoelectric colorimeter with filter no. 626 (Evans Electroselenium Ltd., Harlow, Essex).

Chromatography on Sephadex. In these experiments Sephadex G-25 of 200-400 mesh (fine grade) was used. Very fine particles were removed by sedimentation in water. The gel was then suspended in aq. 70% (v/v) propan-2-ol and the suspension placed under vacuum to remove air before the columns were packed. The solvent employed for elution was deaerated before use. Samples (10-25 μ l.) were taken from alternate fractions of the eluate for ninhydrin determinations.

RESULTS

Production of bacilysin

The production of bacilysin when *B. subtilis* A14 was grown in a New Brunswick fermenter in a Czapek-Dox salts-corn-steep liquor medium is shown in Fig. 1. Most of the antibiotic was formed during a 2 hr. period, at a time when the growth of the culture was still rapid though the phase of exponential multiplication appeared to be nearing its end and when the pH of the medium had fallen from an initial value of 6.8 to one close to 6.0. The maximum yield of bacilysin was about 20 units/ml. However, the complexity of the crude product, as revealed by chromatography and electrophoresis on paper, suggested that purification of the antibiotic would be facilitated if it could be produced in a simpler medium that did not contain the amino acids of corn-steep liquor.

The sulphated ash of corn-steep liquor was found to be as effective as corn-steep liquor itself in stimulating bacilysin production when added to a medium containing Czapek-Dox salts and glucose. Koffler, Knight & Frazier (1947) had reported that

Fig. 1. Production of bacilysin in a glucose-corn-steep liquor-Czapek-Dox salts medium. x, Extinction of culture in Spekker absorptiometer; O, pH; ●, bacilysin (units/ml.).

the effect of corn-steep liquor ash in stimulating the production of penicillin by *Penicillium chrysogenum* could be reproduced by the addition of iron and soluble phosphates to the medium. The effect of iron on the production of bacilysin was therefore studied in the present experiments. In the basic medium containing Czapek-Dox salts and glucose *B. subtilis* A14 did not grow, but both growth and bacilysin production occurred when ferric chloride was added to the medium. The yield of antibiotic increased with the amount of ferric chloride until the latter reached approx. 300mg./ml. and then declined. More rapid growth of the organism and a higher yield of bacilysin resulted from the replacement by ammonium acetate of the sodium nitrate that served as a nitrogen source in the mixture of Czapek-Dox salts. The resulting medium, together with a mixture of trace elements, provided the chemically defined medium described in the Methods section. In this medium the yield of bacilysin was normally between 18 and 20 units/ml. Antibiotic production was similar in inverted T-tubes, indented flasks and the New Brunswick fermenter.

The purification of bacilysin was facilitated if cultures were harvested as soon as possible after the antibiotic titre had reached its maximum. This was normally after 8-10 hr., when the pH of the culture (which had fallen earlier to 6.6) was approaching 7.0. After harvesting the pH was lowered to 3.2 with acetic acid and most of the bacteria were removed by centrifugation. The supernatant was used for the first stage of the purification process.

Purification of bacilysin

In previous work (Newton, 1949) it had been found that bacilysin could be adsorbed from culture fluids on charcoal and eluted with aqueous ethanol containing phosphate buffer. Subsequent experiments showed that the resulting material could be purified further by countercurrent distribution between phenol and an aqueous solution of 50mm-barium acetate, a system in which the partition coefficient of bacilysin was close to 1. However, it appeared that there was some loss of antibacterial activity in this system and that many transfers would be required to resolve some of the components of the mixture. Attempts were therefore made to devise a process of purification that was based on the use of ion-exchange resins.

Preparation and nature of crude concentrates. Preliminary experiments showed that bacilysin was adsorbed on Dowex 50 (X4; H⁺ form) from culture supernatants and eluted at pH 7.1. It was not adsorbed on Amberlite IRC-50 (H⁺ form), and although it was adsorbed from solutions at pH 8.5-pH 9.5 on strongly basic ion-exchange resins [Dowex 1 (X8) and Amberlite IRA-400] it could not be recovered from the latter by elution with N-acetic acid.

For experiments on a preparative scale Dowex 50 was replaced by the cheaper cation-exchange resin Zeo-Karb 225 (SRC5; 14-52 mesh) in the H⁺ form. Culture supernatant was passed through a column of resin whose volume was 4.5% of that of the supernatant used. The flow rate was such that all the fluid had normally entered the column in less than 1 hr. The resin was then washed with 2 bed vol. of 10mN-hydrochloric acid and bacilysin was immediately eluted with 1.3 bed vol. of N-pyridine. The active eluates were concentrated to a small volume *in vacuo* in a rotary evaporator and stored at -20°. The recovery of antibacterial activity at this stage was usually about 50% of that detected in the culture supernatant. Rapid completion of the adsorption and elution process appeared to be important, for in one experiment in which the material remained adsorbed on the resin for 4 hr. no activity was subsequently eluted. Determinations of the weight of solid (dried at 105° *in vacuo*) in the concentrated eluates indicated that the specific activity of the material was 30-40 units/mg.

After the material in the concentrates had been subjected to paper chromatography in butan-1-ol-acetic acid-water, followed by electrophoresis in a perpendicular direction at pH 1.8, a single zone of inhibition was revealed by bioautography. The active compound showed *R_F* 0.43 and its migration towards the cathode was similar to that of glutamic acid. On electrophoresis at pH 4.5 it behaved as though it had no net charge.

In some preparations bacilysin appeared to be a major ninhydrin-positive component. In others, however, the ninhydrin spray revealed the presence of relatively large amounts of glutamic acid, glycine, alanine, valine, tyrosine, phenylalanine, leucine/isoleucine and unidentified compounds, and a number of other substances were visible in ultra-violet light. During further studies it became evident that preparations which contained large quantities of phenylalanine and tyrosine were particularly difficult to purify. It also appeared that the greatest amounts of ninhydrin-positive impurities were present in preparations obtained from cultures which were harvested late and that these impurities had leaked from cells which were no longer in a state of exponential growth. This emphasized the desirability of harvesting cultures and removing the cells as soon as possible after the titre of bacilysin had reached its maximum.

Chromatography on ion-exchange resins. A crude concentrate of bacilysin (50ml.) from 331. of culture

fluid (280000 units) was concentrated further *in vacuo* to 15ml. The pH was then lowered to 3.0 with 6*N*-hydrochloric acid, and the solution was clarified by centrifugation and added to a column of Dowex 50 (X2). Fractions (8.5ml.) were collected with a flow rate of 35ml./hr. The results of one experiment are shown in Fig. 2. The active fractions were combined and the solution was freeze-dried to yield a light-brown powder (285mg.). The recovery of activity from columns of this type was about 25% and the specific activity of the products was 80–90 units/mg.

Part (171mg.) of the product from the column of Dowex 50 (X2) was dissolved in water (1.0ml.), the pH of the solution lowered by addition of acetic acid to 3.8, and the sample applied to a column of Dowex 50 (X8). Fractions (3.0ml.) were collected with a flow rate of 18ml./hr. The results are shown in Fig. 3. The first peak, which contained pigmented material, may represent substances of relatively high molecular weight that were excluded from Dowex 50 (X8). The active fractions (68–81) were combined and the solution was freeze-dried to yield a powder (49mg.) with an activity of 135 units/mg. The recovery of activity from other columns of Dowex 50 (X8) was similar (about 25%) and the specific activity of the products was also about 130 units/mg.

Fig. 2. Chromatography of bacilysin on Dowex 50 (X2; 200–400 mesh) in 0.5 *N*-pyridine-acetate buffer, pH 4.5 (for details see the text). Ninhydrin colour densities represent the extinctions measured in an EEL photoelectric colorimeter when 25 μ l. samples from alternate fractions were assayed by the photometric ninhydrin method. Bacilysin was found by antibacterial assay in fractions 56–66.

Fig. 3. Chromatography on Dowex 50 (X8; 200–400 mesh) in 0.2 *N*-pyridine-acetate buffer, pH 5.0, of crude bacilysin eluted from Dowex 50 (X2) (for details see the text). Samples (50 μ l.) from alternate fractions were used for the measurement of ninhydrin colour densities. Bacilysin was found in fractions 66–81.

Chromatography on Sephadex. Material (about 530mg.) obtained after chromatography on Dowex 50 (X8) was dissolved in 0.6ml. of water, and 1.4ml. of propan-2-ol was added. The solution was applied to a column (30cm. $\times$ 0.9cm. diam.) of Sephadex G-25 (200–400 mesh) packed in 70% (v/v) propan-2-ol. Elution was carried out with 70% (v/v) propan-2-ol, the flow rate being 4ml./hr. and 1ml. fractions being collected. The result is shown in Fig. 4. The active fractions (29–45) were concentrated *in vacuo* in a rotary evaporator and the resulting solution was freeze-dried. The product was washed with acetone–ether (1:1, v/v) and redried to give 171mg. of an off-white powder with an activity of 208units/mg.

Properties of bacilysin

The most active preparation of bacilysin obtained by the process described above showed an activity of 208units/mg. It appeared as a single spot, coloured with ninhydrin, but not with diazotized sulphanilic acid or *p*-dimethylaminobenzaldehyde (Jepson & Smith, 1953), when subjected to chromatography on paper in butan-1-ol–acetic acid–water followed by electrophoresis at pH 1.8, or to electrophoresis at pH 4.5 followed by chromatography.

Fig. 4. Chromatography on Sephadex G-25 in aq. 70% (v/v) propan-2-ol of bacilysin obtained by chromatography on Dowex 50 (X8). Samples (10 μ l.) were taken from alternate fractions for determination of ninhydrin colour densities. Bacilysin was found in fractions 29–45.

Tests for guanidine groups (Jepson & Smith, 1953) and for sulphur (Sjöquist, 1953) were negative. In butan-1-ol–acetic acid–water the product showed an R_F value (0.43) that was slightly greater than that of tyrosine (0.41), and it migrated slightly further than tyrosine towards the cathode on electrophoresis at pH 1.8. It behaved as though it had no net charge on electrophoresis at pH 4.5. A less pure preparation (160units/mg.) contained a minor active component that appeared as a spot with R_F 0.15 in butan-1-ol–acetic acid–water after bioautography with plates seeded with *S. aureus*.

Fig. 5 shows the lytic effect of bacilysin on a culture of *S. aureus* (N.C.T.C. 6571) growing at 37° in Oxoid broth in inverted T-tubes rocked 50 times/min. The broth (in each case 9ml.) was inoculated with 1.0ml. of an overnight culture of the organism. When the extinction, measured in a Spekker absorptiometer, had reached 0.29 a solution of bacilysin (5.2units in 0.02ml.) was added to one tube and a solution of cephalosporin C (8.2 units in 0.1ml.) to another. The extinctions of these cultures and that of a control culture were then read at intervals of about 30min.

DISCUSSION

The presence of ferric iron in the chemically defined medium was apparently essential for optimum growth of the organism and for antibiotic

Fig. 5. Effect of bacilysin and cephalosporin C on the opacity of a growing culture of *S. aureus*. $\times$, Control culture; $\bullet$, culture containing bacilysin (0.52unit/ml.); $\circ$, culture containing cephalosporin C (0.8unit/ml.). The arrow shows the time at which the antibiotics were added.

production. Ferrous iron was ineffective. Antibiotic production occurred when growth of the culture was still rapid, but it does not follow that there is an obligatory linkage between protein and antibiotic synthesis.

The procedures described above for the purification of the bacilysin were developed before anything was known about the nature of the labile structure in the antibiotic, and the reasons for the substantial losses in activity that were sometimes encountered were not understood. However, a survey of the results of a series of experiments indicated that evaporation of aqueous solutions of bacilysin containing relatively high concentrations of pyridine was particularly liable to be accompanied by inactivation. Thus in the preparation of crude concentrates of bacilysin the highest yields (up to 75%) were obtained when the pH of the pyridine eluate from Zeo-Karb 225 was between 7.1 and 7.7 and the lowest yields (about 12%) when it was 8.4. Pyridine-acetate buffers were used for chromatography on Dowex 50 (X2) and Dowex 50 (X8) because of their volatility, but the use of these buffers may have been responsible for some losses of activity during subsequent manipulations. A possible reason for such losses, and for others that may have resulted from a reaction involving anionic centres on the cation-exchange resins, can be given in terms of the chemistry of bacilysin, which is considered in the accompanying paper (Rogers, Lomakina & Abraham, 1965).

The highest overall recovery of apparently pure bacilysin obtained in the present experiments from crude concentrates was 12%. It is probable that this could be raised by modification of the stages involving chromatography on cation-exchange resins. But the reason why the yields obtained

from chromatography on Sephadex G-25 were not greater than 50% is not yet clear.

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