The Use of Acid Alumina and Sephadex LH-20 for the Separation and Characterization of Ethanol-Soluble Peptides Produced by *Bacillus brevis*

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Cell extracts from *Bacillus brevis* (A.T.C.C. 10068), grown with various media, incorporated certain ^{14}C -labelled amino acids that are normally components of tyrothricin into material that was extracted by ethanol from the precipitate formed by adding acid. When this material was separated by paper and silica-gel thin-layer chromatography and paper electrophoresis ^{14}C was located in those regions that also contained gramicidin and tyrocidine. From a study of the properties of the system responsible for the incorporation it was deduced that non-tyrothricin materials were present. It was shown that the methods normally used to characterize tyrothricin do not adequately distinguish between tyrothricin and non-tyrothricin materials. However, a method for separating these materials was devised. This involved elution with ethanol from columns of acid alumina followed by gel filtration on Sephadex LH-20 with dimethylformamide–water solvent. The behaviour of gramicidin and tyrocidine on the Sephadex LH-20 column was examined, and it was concluded that the separation was not caused simply by gel filtration of unassociated molecules. Also, tyrocidine molecules with different amino acid compositions seemed to have different affinities for the Sephadex LH-20 column.

The biosynthesis of tyrocidine catalysed by cell-free extracts of *Bacillus brevis* has been studied by measuring the incorporation of ^{14}C -labelled amino acids into material with properties similar to commercial tyrocidine in acidified salt solutions and methanol or ethanol, and which also behaves like tyrocidine in paper-chromatographic and/or paper-electrophoretic separations. The first reports (Uemura *et al.*, 1963; Okuda *et al.*, 1964*a,b*; Bodley *et al.*, 1964) claimed that the biosynthetic process was similar to that involved in protein biosynthesis. Amino acids were activated via adenylate and incorporated into tRNA, and were then transferred from aminoacyl-tRNA to ribosomes, where the peptide bonds were formed. The specificity for the peptide sequence produced was present in the soluble phase (140000*g* supernatant), and the process was inhibited by pancreatic ribonuclease, puromycin and chloramphenicol. In a later report from the same laboratory (Rao *et al.*, 1968) it was claimed that the above results could not be repeated, and the authors could give no scientific explanation for them.

Meanwhile other workers had shown that the

tyrocidine-biosynthetic process in whole cells was not affected by compounds that inhibited normal protein-biosynthetic mechanisms (Mach *et al.*, 1963), and that the amino acid sequence of tyrocidine produced by cultures was modified by changing the amino acid composition of the medium (Mach & Tatum, 1964; Ruttenberg & Mach, 1966). Likewise the activity of a partially purified tyrocidine-synthesizing enzyme preparation was not affected by puromycin, chloramphenicol or pancreatic ribonuclease (Fujikawa *et al.*, 1966), and the composition of the tyrocidine synthesized by this enzyme preparation was altered by altering the amino acids present during the incubation period (Fujikawa *et al.*, 1968*b*). The details of the mechanisms involved in the biosynthesis of tyrocidine by partially purified enzyme preparations have been reported by Roskoski *et al.* (1970*a,b*), and it is clear that in this case mRNA, aminoacyl-tRNA and ribosomes are not required. However, the maximum rate of synthesis of tyrocidine they obtained with these partially purified preparations was not greater than 1.5 nmol/h per mg of protein. Fujikawa *et al.* (1968*a*) obtained rates of 30 nmol/h per mg of protein with their partially purified preparations, but in crude extracts obtained rates of only 0.1 nmol/h per mg of protein. These rates are much less than those found in the cultures from which the cells for these extracts were obtained. From the results of Fujikawa *et al.* (1968*a*) it is possible to calculate that a minimum rate of 150 nmol

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of tyrocidine/h per mg of protein exists *in vivo*, and this might be only a fraction of the true rate of synthesis by the tyrocidine-synthesizing enzymes, since these enzymes will represent only a small fraction of the total protein present.

An explanation for these large differences in rates is required before one can be certain that the mechanisms now being described by these workers are the only ones or just part of the ones responsible for tyrocidine synthesis *in vivo*. When crude extracts are used to study incorporation of ^{14}C -labelled amino acids into tyrocidine we have found that there is also incorporation into material that is not tyrocidine but that cannot be effectively separated from tyrocidine by the methods usually employed, i.e. acid precipitation, ethanol extraction, paper chromatography, paper electrophoresis or t.l.c. on silica gel. Some of the unconfirmed results could have been affected by incorporation into this non-tyrothricin material.

In the present paper we describe experiments with ^{14}C -labelled tyrothricin-like materials produced by cell extracts of *B. brevis* incubated with ^{14}C -labelled amino acids and the method that we have devised for separating tyrothricin from non-tyrothricin materials. Certain properties of a Sephadex LH-20 column that we developed in our attempts to characterize tyrothricin are also described. In this way we hope to warn others of the pitfalls involved in not rigorously establishing the identities of materials such as tyrocidine whose synthesis by cell extracts is detected only by methods involving the use of radioactive isotopes.

Materials and Methods

Chemicals

The ^{14}C -labelled amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and had the following specific radioactivities: [1- ^{14}C]glycine, 41.4 Ci/mol; L-[U- ^{14}C]glutamine, 41.3 Ci/mol; L-[U- ^{14}C]leucine, 6.5 Ci/mol; DL-[1- ^{14}C]phenylalanine, 48 Ci/mol; L-[U- ^{14}C]phenylalanine, 12.6 Ci/mol; L-[U- ^{14}C]serine, 153 Ci/mol; DL-[methylene- ^{14}C]tryptophan, 56 Ci/mol; L-[U- ^{14}C]tyrosine hydrochloride, 5.5 Ci/mol; DL-[1- ^{14}C]valine, 4.76 Ci/mol. Specific radioactivities of amino acids different from these are described in the text. The 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were purchased from Packard Instrument Co., Wembley, Middx., U.K. Tyrocidine and tyrothricin were purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., gramicidin was from Sigma (London) Chemical Co., London S.W.6, U.K., chloramphenicol was from Parke-Davis, Hounslow, Middx., U.K., puromycin hydrochloride was from American Cyanamid Co., New York, N.Y., U.S.A., pancreatic ribonuclease was from Koch-Light Laboratories

Ltd., Colnbrook, Bucks., U.K., ATP (disodium salt) pyruvate kinase and phosphoenolpyruvic acid were from Boehringer Corp. (London) Ltd., London W.5, U.K., bacteriological peptone was from Evans Medical Ltd., Speke, Liverpool, U.K., and acid alumina was from M. Woelm, Eschwege, Germany.

NN-Dimethylformamide (laboratory reagent; Fisons Scientific Apparatus, Loughborough, Leics., U.K.), was distilled once, the fraction that boiled between 150° and 152°C being collected. Commercial ethanol was refluxed for 3 h with sodium and distilled once, the fraction boiling at 78°C being collected. All other reagents were of A.R. grade where possible.

Organism, growth conditions and preparation of cell extracts

Organism. The organism was derived from a strain of *B. brevis* (A.T.C.C. 10068) supplied by Dr. B. Mach (Mach *et al.*, 1963).

Medium and growth conditions. Stock cultures were freeze-dried in horse serum from 16h-old nutrient broth (Oxoid) cultures and stored *in vacuo*. These were reconstituted in a medium (PYN) adjusted to pH 7.0 and containing peptone (Evans), 1.0 g/100 ml, yeast extract (Difco), 0.3 g/100 ml, and NaCl, 0.5 g/100 ml, then they were grown overnight at 37°C and streaked on medium with the same composition but solidified with 2% (w/v) of agar. A single colony was selected and grown on solid medium. Spore suspensions were prepared by inoculating 100 ml quantities of asparagine, glycerol minimal (AGM) medium (Mach *et al.*, 1963), that were dispensed in 500-ml wide-necked flasks and shaken at 37°C on a reciprocating shaker. Cultures were examined with a phase-contrast microscope, and when spore formation was optimum (2–3 days) 2 ml portions were stored frozen (–15°C) in sterile tubes. For the preparation of extracts the organism was grown in either PYN medium dispensed in 100 ml quantities in Glaxo penicillin flasks or in 6-litre batches of AGM medium in 10-litre flasks. PYN medium was inoculated with a sufficient quantity of spore suspension, previously heated at 85°C for 5 min, and well dispersed with the help of a Whirlimixer (Fisons), so that the organism was in the stationary phase of growth (approx. 1.7 mg dry wt./ml) after about 16 h at 37°C with non-shaking conditions [6 litres of AGM medium at 37°C, vigorously agitated with a stream of sterile air, was inoculated with 300 ml of growing culture (0.2–0.3 mg dry wt./ml, grown in the same medium but dispensed in 100 ml quantities in 500-ml wide-necked flasks and shaken)]. After 16 h a cell density of about 0.35 mg dry wt./ml was obtained, and this increased further to about 0.70 mg dry wt./ml by 24 h. Growth was measured spectrophotometrically at 600 nm and expressed as mg dry wt. after conversion from a calibration graph. Where

necessary, cultures were diluted with distilled water so that E_{600} did not exceed 0.3, and cells were harvested either by centrifugation at 12000g for 2min at 4°C or in a rotor-driven Sharples centrifuge.

Cell extracts. The cell paste was washed with cold (5°C) 5mM-MgCl₂, pH7.0, and transferred to a frozen modified (Sutton & King, 1966) Hughes press. It was left at -15°C for 1 h, before being crushed and mixed with half its weight of polishing alumina (grade 3/50; Griffin and George Ltd., Manchester, U.K.). The mixture was ground in a pre-chilled (2°C) mortar with a pestle and dispersed in cold buffer (4.0ml/g of cell paste), which was slowly added during grinding. Alumina, cell debris and large particulate matter were removed by centrifugation at 25000g for 15min at 0°C. The clear yellow supernatant was carefully decanted and stored at 2°C until required for incorporation studies. Extracts prepared in this way contained about 15mg of protein/ml. Crystallized bovine plasma albumin (Armour Laboratories, Eastbourne, Sussex, U.K.) dried over P₂O₅ was used as standard for protein determinations by the method of Stickland (1951), and where necessary a correction was made for the presence of tris buffer.

Incubation conditions

Mixture A. A final volume of 2.5ml at pH7.8 contained: tris-HCl buffer, pH7.9, 12.5μmol; MgCl₂, 7.5μmol; GSH, 20μmol; ATP, 5μmol; phosphoenolpyruvate, 5μmol; L-asparagine, L-aspartic acid, L-glutamic acid, L-ornithine, L-proline and D-tryptophan, 1.0μmole each; D-leucine, L-phenylalanine and D-valine, 2.0μmol each; L-leucine and L-valine, 3.0μmole each; L-tyrosine, 1.2μmol; pyruvate kinase, 20μg; cell extract, 1.0ml. The 1.0ml of cell extract contained: tris-HCl buffer, pH7.9, 50μmol; MgCl₂, 5μmol; GSH, 10μmol.

Mixture B. A final volume of 3.0ml at pH7.5 contained: tris-HCl buffer, pH7.5, 300μmol; MgCl₂, 30μmol; ATP, 6μmol; DL-phenylalanine, DL-tryptophan and DL-valine, 1.0μmol each; L-alanine, L-asparagine, L-glutamic acid, glycine, L-leucine, L-ornithine, L-proline and L-tyrosine, 0.5μmol each; cell extract, 1.0ml. The 1.0ml of cell extract contained: tris-HCl buffer, pH7.5, 50μmol; MgCl₂, 5μmol; 2-mercaptoethanol, 15μmol.

In both cases pH adjustments were made with KOH while the pH was checked with a glass electrode. For incorporation studies 1μCi of a ¹⁴C-labelled amino acid was added to a centrifuge tube incubated at 37°C and containing the incubation mixture from which the appropriate unlabelled amino acid and extract had been omitted. Cell extract was added to start the reaction, which was stopped after a suitable time by adding either (mixture A) sufficient 2M-HCl to lower the pH to 3.5 followed by heating for 5min in a boiling-water bath, or (mixture B) trichloro-

acetic acid solution to give a final concn. of 5% (w/v). Modifications to these conditions are described in the text.

Extraction of tyrothricin-like material and measurement of ¹⁴C-labelled amino acid incorporated

From incubation mixtures. Method A. After mixture A had been treated with HCl and boiled, it was centrifuged for 10min at 5000g, and the sedimented material was washed three times with 5ml of 2% (w/v) NaCl and then resuspended in 5ml of ethanol. After being left overnight the suspension was centrifuged; the supernatant was retained, and the sediment was resuspended in 2ml of ethanol for a further 2h and again centrifuged. The combined ethanol supernatants were concentrated to an exact volume by evaporation at between 50° and 60°C under a stream of N₂. For measurement of incorporation of ¹⁴C-labelled amino acids, portions of the above solution were dried with an i.r. lamp on tared aluminium planchets and their radioactivities counted.

Method B. To mixture B that had been treated with trichloroacetic acid was added 0.5mg of commercial tyrocidine dissolved in 0.05ml of 90% (v/v) ethanol. The precipitate was well dispersed with a glass rod and the mixture was centrifuged at 1500g for 5min. The pellet was dispersed in 5% (w/v) trichloroacetic acid, then it was washed twice in 2% (w/v) NaCl and redispersed in 4ml of ethanol-0.2M-HCl (9:1, v/v). After being left overnight the suspension was centrifuged, the supernatant was retained, and the sediment was resuspended in 2ml of ethanol and recentrifuged. The combined ethanol supernatants were evaporated to dryness at 40°C under a stream of N₂. The residue was dissolved in 1.3ml of 90% ethanol; samples were dried on aluminium planchets with an i.r. lamp and their radioactivities were determined.

From cells grown with AGM medium. Cell paste from 6 litres of AGM medium was resuspended in 200ml of fresh AGM medium, trichloroacetic acid was added to a final concn. of 5% (w/v), then the mixture was heated in steam for 5min, before being cooled and centrifuged at 10000g for 10min; the sedimented material was washed twice with 40ml of 1% (w/v) NaCl. Tyrothricin was extracted by resuspending the sediment in 40ml of ethanol-0.2M-HCl (9:1, v/v) for 2h, then centrifuging the suspension at 10000g for 20min; the sediment was re-extracted with 30ml of ethanol overnight and the solution was centrifuged. The ethanol supernatants were combined and concentrated to 15ml by evaporation *in vacuo* at 50°C. The dark-brown liquid was poured into 150ml of 1% (w/v) NaCl and kept for 3 days before being centrifuged. The supernatant was decanted and the residue (tyrothricin) was dissolved in 10ml of ethanol.

Methods for separating and detecting ethanol-soluble peptides

Paper chromatography. Samples dissolved in ethanol were applied to Whatman no. 1 paper, which was equilibrated and developed in an ascending manner with either the upper phase of cyclohexane-propan-2-ol-water (2:2:1, by vol.) (Uemura *et al.*, 1963) or butan-1-ol-acetic acid-water (12:3:5, by vol.). Papers were air-dried, and gramicidin and tyrocidine were detected either by dipping the paper in 7M-HNO₃ (Okuda *et al.*, 1963), when yellow spots appeared on a white background (detection limits 5 µg of tyrocidine and 3 µg of gramicidin), or by dipping the paper in or spraying it with 0.2% (w/v) ninhydrin in dry acetone and heating it at 60°C for 30 min; tyrocidine gave a blue-grey spot and gramicidin a grey spot (detection limit about 100 µg in each case).

T.l.c. Samples on Eastman Chromagram silica-gel sheets type K 301V were separated with butan-1-ol-acetic acid-water (4:1:1, by vol.) for 4 h in the Eastman chamber-plate apparatus. Dried sheets were sprayed with either 1% (v/v) ninhydrin in 70% ethanol and dried with hot air (detection limit about 30 µg of peptide), or 0.004% (w/v) fluorescein in ethanol followed by examination with a u.v. lamp (253.7 nm average emission), when peptides appeared as dark spots (detection limit about 10 µg of peptide) (Bartley, 1969).

Paper electrophoresis. Samples were subjected to electrophoresis on Whatman 3MM paper with 50% (v/v) acetic acid (Uemura *et al.*, 1963) for 2–6 h at a potential gradient of 30 V/cm with a flat-bed high-voltage apparatus cooled by running water. Peptides were located with 7M-HNO₃.

Acid alumina (Mach *et al.*, 1963). Acid alumina (Woelm) was mixed with 90% (v/v) ethanol and the slurry was poured into a 1 cm-diameter glass tube fitted with a sintered-glass disc (porosity 3) to give a depth of alumina of about 1 cm. Samples dissolved in 90% (v/v) ethanol (up to 2 ml) were applied and eluted with 2 ml of 90% (v/v) ethanol. The rate of elution was increased by applying pressure with N₂. Of the tyrocidine (1 mg/ml) 100% and of the gramicidin (1 mg/2 ml) 97% were eluted, whereas ¹⁴C-labelled phenylalanine, leucine, tryptophan, arginine and glutamic acid were retained.

Column chromatography with Sephadex LH-20 (Bartley, 1969). Mach & Tatum (1964) used long (0.9 cm × 150 cm) columns of Sephadex G-25 to fractionate tyrocidine, which had been previously separated from gramicidin by paper electrophoresis, into its constituent tyrocidines A, B, C and D with 10% (v/v) acetic acid solvent. The behaviour of peptides produced by partial degradation of tyrocidine C, and of amino acids, showed that the order of elution did not correspond to their molecular size,

but that molecules of similar molecular size were eluted in the order corresponding to their relative tryptophan contents (Ruttenberg *et al.*, 1965). Attempts in our laboratories to separate tyrocidine from gramicidin and other ethanol-soluble peptides on Sephadex G-25 with various concentrations of acetic acid proved unsuccessful, and it seemed that part of the difficulty was due to the tendency of tyrocidine molecules to associate even in a 50% (v/v) acetic acid solvent (Craig & King, 1955). Ruttenberg *et al.* (1966) found that tyrocidine has less tendency to associate in dimethylformamide than in acetic acid solutions, and the tendency was further suppressed at higher concentrations of dimethylformamide. Sephadex LH-20 is an alkylated cross-linked dextran, with lipophilic properties, that is readily penetrated by dimethylformamide and gives good fractionation of hydrophobic materials according to their molecular size in the molecular weight range 100–4000 (Pharmacia Fine Chemicals). Consequently we attempted to fractionate tyrothricin and other ethanol-soluble peptides on Sephadex LH-20 with dimethylformamide solvent.

Sephadex LH-20 that had been stirred for at least 4 h with aq. 90% (v/v) dimethylformamide was poured into a 2 cm-diameter glass column, and packed by gravity to a height of 13 cm; the column was then washed through with aq. 90% (v/v) dimethylformamide for 4 h at a flow rate of 9 ml/h. A mixture of commercial gramicidin (1.95 mg) and tyrocidine (2.05 mg) dissolved in aq. 90% (v/v) dimethylformamide was applied and eluted with the same solvent. Fractions of volume 1 ml were collected and, after dilution with 2 ml of solvent, their *E*₂₈₀ values were determined. Gramicidin and tyrocidine were well separated (Fig. 1), and, by using the *E*_{1cm}^{0.1%} values 11.5 and 5.0 respectively, it was calculated that 91% of the gramicidin and 92% of the tyrocidine had been recovered. Further experiments established the exclusion limit by using crystalline dinitrophenyl-insulin (mol.wt. 5900), the inclusion limit by using acetone (mol.wt. 58) and that both peptides were eluted before added ¹⁴C-labelled amino acids. A 1 ml portion of the tyrothricin fraction obtained from cells grown with AGM medium was evaporated to dryness at 55–60°C under a stream of N₂ and then it was dissolved in 0.4 ml of aq. 90% (v/v) dimethylformamide, and 0.3 ml was resolved on the same column (Fig. 2). Material in fractions 18 and 19 was eluted at the same position as commercial gramicidin and also gave the same *R_F* on t.l.c. as commercial gramicidin. Material in fractions 23–27 was eluted slightly ahead of commercial tyrocidine (fractions 26–29), but nevertheless gave an *R_F* value on t.l.c. identical with that of commercial tyrocidine. The quantity of tyrocidine present in the sample was about ten times that used previously (Fig. 1). In an experiment with a

Fig. 1. Elution pattern of a mixture of gramicidin and tyrocidine from a column of Sephadex LH-20

Gramicidin (1.95mg) and tyrocidine (2.05mg) were eluted from a column (13cm×2cm diam.) of Sephadex LH-20 with aq. 90% dimethylformamide, 1.0ml fractions being collected. Each fraction was diluted to 3.0ml with solvent, and its E_{280} was determined. Gramicidin (A) and tyrocidine (B) were eluted in the peaks indicated. The positions of elution of dinitrophenyl-insulin (C), [^{14}C]phenylalanine (D) and acetone (E) are also indicated.

smaller sample, material was again eluted in tubes 18 and 19, but the second peak was found in tubes 25–28 (Fig. 2). This dependence of the exact position of elution on the concentration of tyrocidine was confirmed with smaller concentrations of tyrocidine. The dimensions of the column used were not critical, and in some experiments a column of dimensions 26.5cm×1 cm diam. was used.

Fig. 2. Elution patterns of tyrothricin containing two different amounts of tyrocidine from a column of Sephadex LH-20

Tyrothricin containing either 21 mg (○; right-hand ordinate) or approx. 4mg of tyrocidine (△; left-hand ordinate) was eluted from a column (13cm×2cm diam.) of Sephadex LH-20 with aq. 90% dimethylformamide. For the 21mg sample fractions were diluted to give E_{280} values that could be read on the spectrophotometer, and the E_{280} values shown were calculated. For the 4mg sample E_{280} values were determined as indicated in Fig. 1 legend.

Detection and determination of radioactive materials

By solid-scintillation counting. The radioactivity of material that had been dried on to aluminium planchets under an i.r. lamp was counted with the Panax system Co 112/x PD-02, which consisted of an anthracene crystal detector and low-noise photomultiplier tube.

The operating external high tension was 850V with a discriminator bias of 5 mV and a dead-time of 20 μs . Efficiency of counting ^{14}C β -emission was about 20% and was determined by plating out portions of freshly opened samples of ^{14}C -labelled amino acids dissolved in a known quantity of sterile water. As the weight of material on any planchet did not exceed 0.5mg, corrections for self-absorption were unnecessary.

By liquid-scintillation counting. A 10ml volume of scintillation fluid [700ml of toluene, 300ml

of ethanol, 4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Bernlohr & Novelli, 1963)] was added to glass vials that contained samples in water (not more than 0.1 ml), aq. 90% (v/v) dimethylformamide (not more than 0.5 ml), ethanol and pieces of chromatography paper or silica-gel thin layers on plastic backing. Counting of radioactivity was done with a Packard Tri-Carb model 3310 liquid-scintillation spectrometer operated automatically in conjunction with a Munroe print-out. The amplifier gain setting was 8% and the lower and upper discriminator settings were 50 and 1000 respectively. Although ethanol in the solvent caused quenching the solvent could be used with all types of samples and the quenching characteristics did not vary significantly with the different samples used.

On thin-layer and paper chromatograms and electrophoretograms. For quantitative measurements of such samples, the regions of paper containing the radioactivity were cut into small pieces and extracted with ethanol. The ethanol solutions were then dried on to aluminium planchets and their radioactivities counted as described above. For direct quantitative measurement and location of ^{14}C -labelled material, strips (1 cm $\times$ 0.5 cm long) of plastic-backed thin-layer chromatograms cut with a razor blade and strips of paper chromatograms (3 cm wide $\times$ 1 cm long) were placed directly in glass vials and their radioactivities counted by liquid-scintillation counting. Radioautography was done with Kodak Blue Band X-ray film.

Other methods

Hydrolysis of peptide material. Samples (not more than 2 mg) and 1 ml of 6M-HCl were sealed *in vacuo* in Pyrex tubes and heated for 16 h at 110°C. After being cooled to 4°C the tubes were opened, and the contents were washed into a flask with water and dried *in vacuo* at 60°C. The residue was resuspended in water and re-evaporated two or three times to ensure removal of HCl. Finally the residue was dissolved in a small quantity of aq. 10% (v/v) propan-2-ol for chromatography.

Paper chromatography of amino acids and hydrolysates of peptide material. Samples in aq. 10% (v/v) propan-2-ol were applied to 25 cm $\times$ 25 cm sheets of Whatman no. 1 paper which were developed in an ascending manner with butan-1-ol-acetic acid-water (12:3:5, by vol.) on stainless-steel Aimer frames in one dimension for 16 h. After drying for 8 h at room temperature in a stream of air, phenol solvent [120 ml of water mixed with 500 g of phenol, and 1 ml of aq. NH_3 solution (sp.gr. 0.88) added to each 200 ml of mixture just before use] was used for 16 h in the second dimension.

Chromatograms were dried in air and dipped in

acetone to remove excess of phenol, then were dried again and sprayed with 1% (w/v) ninhydrin in 70% (v/v) ethanol either immediately or after radioautography. Colours were developed by heating the chromatograms at 65°C for 20 min.

Results

Holme (1965) studied different conditions of growth for *B. brevis* (A.T.C.C. 10068) and found that maximum production of tyrothricin occurred in a peptone-yeast extract-NaCl medium (PYN) under static conditions. Up to 185 mg of tyrothricin/l of medium was produced during a period of 20 h. The maximum rate of tyrothricin production occurred towards the end of the exponential growth, while growth increased from 1.0 to 1.4 mg/ml. When extracts (15 mg of protein/ml) from cells harvested towards the end of this period were incubated with mixture A containing L- ^{14}C phenylalanine they accumulated ^{14}C -labelled material that behaved like tyrothricin during extraction procedure A. A boiled extract incubated for 1 h produced only 16% of the incorporation obtained with the normal extract and the amount did not increase with time. Separation by paper chromatography of samples of the tyrothricin-like material formed by normal cell extracts followed by radioautography showed one spot at the solvent front, corresponding to the position of gramicidin, and one just away from the origin, corresponding to the position of tyrocidine but streaking a little towards the solvent front and away from the tyrocidine region. Electrophoresis of a sample followed by radioautography again gave two spots, one moving slightly towards the anode and corresponding to the position of gramicidin, the other moving towards the cathode and overlapping the region corresponding to the position of tyrocidine. Elution of the radioactive regions of the electrophoretogram corresponding to the position of gramicidin and tyrocidine gave 31 and 48% respectively of the total radioactivity applied to the paper.

In one experiment increasing the amount of protein present in the 2 h incubation from 12 to 22 mg with 2.0 μCi of ^{14}C phenylalanine (6.8 Ci/mol) resulted in an increased incorporation from 11350 to 20800 c.p.m., and in another experiment doubling the amount of radioactive isotope gave a 1.75-fold increase in incorporation, in both cases into material extracted by method A. The maximum rate of incorporation of ^{14}C phenylalanine obtained at this time was calculated to be about 0.03 nmol/h per mg of protein, which represented about 0.25% of the added ^{14}C phenylalanine. Despite this rather low total incorporation, it seemed reasonable to assume that both gramicidin and tyrocidine were being synthesized by the cell extract. However, Holme (1965)

found that when [^{14}C]glycine was added to cells growing in AGM medium radioactivity was present in the material that was extracted by method A, and, when this material was separated by both paper chromatography and electrophoresis, radioautography showed that ^{14}C was present in regions corresponding to the positions of gramicidin and tyrocidine. Cell extracts from *Escherichia coli* prepared and incubated in the same way as those from *B. brevis* also incorporated L-[^{14}C]phenylalanine into material that could be extracted by method A. However, the total incorporation obtained with the same amount of protein was only one-third of that found with *B. brevis* cell extracts. As glycine is not a recognized component of tyrocidine and *E. coli* would not be expected to synthesize tyrothricin, material that is not tyrothricin must also be extracted by method A.

When $1\mu\text{Ci}$ of L-[^{14}C]proline (12.6Ci/mol) was added and the amount incorporated by extracts into material extracted by method A was compared with that obtained with L-[^{14}C]phenylalanine, in 2h 14 times more phenylalanine than proline was incorporated. This value was even higher when phenylalanine incorporation was compared with that from $1\mu\text{Ci}$ of L-[^{14}C]aspartic acid (4.2Ci/mol) and $1\mu\text{Ci}$ of L-[^{14}C]glutamic acid (3.3Ci/mol). Omission of added amino acids, ATP and/or the ATP-generating system from the incubation mixture A had little or no effect on the amount of L-[^{14}C]phenylalanine incorporated. The inclusion of ribonuclease (20 $\mu\text{g}/\text{ml}$) increased incorporation by 25%, whereas chloramphenicol (25 $\mu\text{g}/\text{ml}$) and puromycin (50 $\mu\text{g}/\text{ml}$) had no effect. Also, on two occasions incorporation of L-[^{14}C]phenylalanine was not diminished by the addition of up to 7 μmol of unlabelled L-phenylalanine to the incubation mixture. These results made us suspect the purity of the L-[^{14}C]phenylalanine, but when this was checked by paper chromatography after the addition of carrier unlabelled phenylalanine there was an exact coincidence of the radioactive areas shown by radioautography and the area showing the presence of ninhydrin-positive material. When the ^{14}C -labelled tyrothricin-like material from an incubation with [^{14}C]phenylalanine was hydrolysed and separated by paper chromatography, radioautography showed the presence of ^{14}C only in the region corresponding to the position of phenylalanine. It was still possible that D-[^{14}C]phenylalanine was present in the L-[^{14}C]phenylalanine sample and that it was the D-[^{14}C]phenylalanine that was being incorporated. This explanation was not tested.

At this stage, which was before the use of acid alumina and Sephadex LH-20, we concluded that, although extracts from cultures of *B. brevis* that were actively synthesizing tyrothricin on PYN medium might synthesize tyrothricin, the measured incor-

poration of ^{14}C -labelled amino acids was also into material other than tyrothricin.

Incorporation by extracts from cells grown with AGM medium

Because of the difficulties encountered by Holme (1965) in his attempts to prepare cell extracts that would actively synthesize tyrocidine, Walker (1971) used extracts from cells growing with AGM medium. Mach *et al.* (1963) had established that the organism produced tyrothricin with a maximum rate of synthesis towards the end of exponential growth, and this had been confirmed by Bartley (1969).

An extract from cells grown with AGM medium was incubated in mixture B for 0.5h with DL-[^{14}C]phenylalanine (5Ci/mol). Material extracted by method B contained three times as much ^{14}C as did the material extracted after incubation with DL-[^{14}C]phenylalanine and boiled extract. L-[U- ^{14}C]Tyrosine

Fig. 3. Chromatography on a column of Sephadex LH-20 of commercial tyrocidine and material extracted with ethanol from a trichloroacetic acid precipitate from an incubation mixture B containing DL-[^{14}C]phenylalanine and an extract from *B. brevis* cells grown with AGM medium

Commercial tyrocidine and the extracted material were chromatographed on a column (26.5cm $\times$ 1cm diam.) of Sephadex LH-20, and 0.5ml fractions were collected. Half of each fraction was taken for counting of radioactivity by liquid-scintillation counting; the other half was diluted with 3.0ml of solvent and its E_{280} value determined. ●, ^{14}C radioactivity; ○, E_{280} .

was also incorporated by a normal cell extract. However, when ATP or amino acids were omitted from the incubation mixture incorporation of [^{14}C]-phenylalanine was not affected.

Part of the material extracted from an incubation mixture with DL-[1- ^{14}C]phenylalanine was separated by paper chromatography with the solvent butan-1-ol-acetic acid-water (12:3:5, by vol.). The strip of paper was cut into pieces and the distribution of ^{14}C was determined by liquid-scintillation counting. Of the added radioactivity 85% was recovered, and 95% of this was present in the region coinciding with and just behind the solvent front. This region overlapped that containing tyrocidine (R_F 0.87) and gramicidin (R_F 0.96). A portion was separated on a column (26.5 cm $\times$ 1 cm diam.) of Sephadex LH-20. Of the added radioactivity 98% was recovered, and most of this was present in a broad peak that slightly overlapped the region containing tyrocidine (Fig. 3). Another portion was run through a column of acid alumina, but only 8% of the added radioactivity was recovered in the eluent. Material that had been eluted from acid alumina was dried, redissolved in aq. 90% (v/v) dimethylformamide and run through a Sephadex LH-20 column as described above. Most of the radioactivity was eluted after tyrocidine (Fig. 4). Consequently we concluded that not even the 8% of the initial incorporated radioactivity that was eluted from acid alumina with ethanol was in tyrothricin.

Material extracted by method B from incubation mixtures containing L-[U- ^{14}C]tyrosine also behaved differently from tyrocidine on silica-gel thin-layer and column chromatography on Sephadex LH-20. When the distribution of radioactivity on the thin layer was determined by liquid-scintillation counting, 85% of the added radioactivity was recovered, and most of this was in the region just behind the solvent front that also contained gramicidin (R_F 0.95), but there was virtually none in the region that contained tyrocidine (R_F 0.48). After chromatography on the Sephadex LH-20 with added tyrocidine most of the radioactivity was eluted before tyrocidine, some being in the region of gramicidin (Fig. 5). However, when a sample was run through a column of acid alumina only 14% of the added radioactivity was eluted with ethanol. Hence materials extracted from incubations with different amino acids did not behave like tyrothricin or some other single compound. Although no further attempts were made to characterize these materials, it was established in separate experiments that L-[U- ^{14}C]leucine, L-[U- ^{14}C]lysine, DL-[1- ^{14}C]valine, and DL-[methylene- ^{14}C]tryptophan were also incorporated into them, whereas L-[U- ^{14}C]-proline and L-[U- ^{14}C]glutamine were not. The maximum rate of incorporation of any amino acid into these materials was obtained with DL-[1- ^{14}C]-phenylalanine and was about 0.03 nmol/h per mg of protein.

Fig. 4. Chromatography on a column of Sephadex LH-20 of material prepared as for Fig. 3 and then eluted from a column of acid alumina with ethanol

Conditions other than the elution from acid alumina were as indicated in Fig. 3 legend. ●, ^{14}C radioactivity; ○, E_{280} .

Fig. 5. Chromatography on a column of Sephadex LH-20 of material prepared as described for Fig. 3 except that the labelled amino acid in the incubation mixture was L-[U- ^{14}C]tyrosine

Conditions apart from the replacement of DL-[1- ^{14}C]phenylalanine by L-[U- ^{14}C]tyrosine in the incubation mixture were as indicated in Fig. 3 legend. ●, ^{14}C radioactivity; ○, E_{280} .

Incorporation of amino acids into tyrocidine

[^{14}C]Tyrocidine production from ^{14}C -labelled amino acids in extracts from cells grown with PYN medium under static conditions was looked for by using the modified procedure for isolating tyrocidine and checking its identity. To try to promote the synthesis of tyrocidine, mixture B was modified by adding only those amino acids that are recognized constituents of tyrocidine, i.e. $1\mu\text{mol}$ each of L-asparagine, L-glutamine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tyrosine and L-valine. Tryptophan was omitted because it was intended to use [^{14}C]phenylalanine and [^{14}C]tyrosine for the incorporation studies.

Cells were grown to four different culture densities, and extracts were prepared and immediately frozen. The following day thawed extract was added to the modified mixture B with $1\mu\text{Ci}$ of DL-[$1\text{-}^{14}\text{C}$]phenylalanine (5Ci/mol), and material was extracted by method B after stoppage of the reaction of one duplicate sample immediately (zero time) and the other after 60 min of incubation. The results (Table 1) confirmed that phenylalanine was incorporated, but the amount of incorporation was different with extracts from cells harvested at different densities. When samples of the extracted materials were run through columns of acid alumina most of the radioactivity was adsorbed (Table 1). The ^{14}C -labelled material eluted from incubations with extracts from cells harvested with culture densities of 0.78 and

0.96 mg dry wt./ml were dried, dissolved in aq. 90% (v/v) dimethylformamide and run through a column ($26.5\text{cm} \times 1\text{cm}$ diam.) of Sephadex LH-20. Determination of the distribution of ^{14}C and E_{280} in the fractions showed that in both cases most of the ^{14}C -labelled material was eluted with tyrocidine (Fig. 6) and was not eluted in the region corresponding to that shown in Fig. 4.

In a separate experiment an extract, prepared from cells harvested at a culture density of 0.9 mg dry wt./ml, that had been immediately frozen, thawed next day and incubated with modified mixture B and $1\mu\text{Ci}$ of L-[^{14}C]tyrosine (5Ci/mol) incorporated 0.31nmol of tyrosine/h per 15mg of protein into material extracted by method B. When this material was run through a column ($26.5\text{cm} \times 1\text{cm}$ diam.) of Sephadex LH-20 and the distribution of radioactivity and E_{280} was determined the pattern was almost identical with that shown in Fig. 5. Also, when another portion was run through a column of acid alumina and the radioactivity in the eluant was determined it was calculated that only 0.012nmol of tyrosine/h per 15mg of protein was incorporated into the material, more than 96% of the radioactivity being adsorbed.

Because material extracted from incubation mixtures with [^{14}C]phenylalanine could pass through a column of acid alumina and then be eluted from a Sephadex LH-20 column in the same position as tyrocidine we studied the effects on incorporation of

Table 1. *Incorporation of DL-[$1\text{-}^{14}\text{C}$]phenylalanine by cell extracts from B. brevis cells grown to different culture densities in PYN medium*

Extracts were prepared from cells grown to different densities in PYN medium and frozen at -15°C overnight. Two 1.0ml portions of each thawed sample were each added to 2ml of incubation mixture containing tris-HCl buffer, pH 7.5 ($300\mu\text{mol}$), MgCl_2 ($30\mu\text{mol}$), ATP ($6.0\mu\text{mol}$), L-asparagine, L-glutamine, L-leucine, L-ornithine, L-proline, L-tyrosine and L-valine ($1.0\mu\text{mol}$ each) and DL-[$1\text{-}^{14}\text{C}$]phenylalanine (5Ci/mol) ($0.2\mu\text{mol}$). Trichloroacetic acid was added to one sample of each pair immediately (zero time) and to the other after 60 min, in each case to give a final concentration of 5% (w/v). To each sample was added 0.5mg of commercial tyrocidine, and material was extracted from the sedimentable material with ethanol by method B (see the text). The ^{14}C present in each of these fractions was measured in samples ('before acid alumina'), and other samples were run through columns of acid alumina and the ^{14}C present in the eluted material was determined ('after acid alumina').

Cell density (mg dry wt./ml)	Protein added to incubation (mg dry wt./ml)	Incubation time (min)	Total DL-[^{14}C]phenylalanine incorporated (nmol)	
			Before acid alumina	After acid alumina
0.61	14.5	60	0.060	0.005
		0	0.045	0.004
0.70	15.0	60	0.260	0.083
		0	0.050	0.005
0.96	16.0	60	0.540	0.140
		0	0.060	0.006
1.18	15.5	60	0.184	0.025
		0	0.050	0.007

many alterations to the incubation conditions, and devised conditions in which cell extracts would catalyse the incorporation of all the tyrocidine

Fig. 6. Chromatography on a column of Sephadex LH-20 of material produced during a 60 min incubation period and extracted as described in Table 1 and then eluted from an acid alumina column with ethanol

B. brevis cells were harvested at a culture density of 0.96 mg dry wt./ml. Conditions for chromatography and determination of the ¹⁴C radioactivity (●) and the E₂₈₀ (○) of the fractions were as indicated in Fig. 3 legend.

amino acids into tyrocidine. The results of some of these modifications have been described (Hodgson & Walker, 1969). The mixture finally chosen was as follows. A final volume of 3 ml at pH 7.5 contained: ATP, 30 μmol; MgCl₂, 30 μmol; L-asparagine, L-glutamine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tyrosine and L-valine, each 1.0 μmol; ¹⁴C-labelled amino acid, 1 μCi; cell extract, 1 ml. The cell extract contained: tris-HCl buffer, pH 7.5, 50 μmol; MgCl₂, 5 μmol; 2-mercaptoethanol, 15 μmol; protein, about 15 mg. Extracts were prepared as quickly as possible from cells harvested at a density of 1.1 mg dry wt./ml and were immediately added to the mixture just described, which had been kept at 37°C for about 2 min. After 1 h the reaction was stopped and the ¹⁴C content of material that could be extracted by method B and then eluted from acid alumina was determined. The quantities of the various amino acids that had been incorporated into these materials were then calculated and the results are shown in Table 2. All the amino acids tested that are recognized constituents of tyrocidine were incorporated, and in every case more than 80% of the ¹⁴C-labelled material extracted by method B was eluted from columns of acid alumina. Glycine and serine, which are not recognized constituents of tyrocidine, were not incorporated to any significant extent. In some cases the material that had been eluted from the acid alumina was dried and run on a column (26.5 cm × 1 cm diam.) of Sephadex LH-20. The distribution of ¹⁴C and E₂₈₀ in the fractions is described in Fig. 7 for material derived from an incubation with L-[U-¹⁴C]phenylalanine. A similar

Table 2. Incorporation of ¹⁴C-labelled amino acids into tyrocidine-like material by cell extracts from *B. brevis* cells grown in PYN medium

An extract was prepared from cells grown in PYN medium to a density of 1.1 mg dry wt./ml, and 1.0 ml portions were each immediately added to 2 ml of incubation mixture at 37°C containing, at pH 7.5, ATP (30 μmol), MgCl₂ (30 μmol), L-asparagine, L-glutamine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tyrosine and L-valine (each 1.0 μmol) and 1 μCi of the indicated ¹⁴C-labelled amino acid. After 1 h trichloroacetic acid was added to a final concentration of 5% (w/v), 0.5 mg of commercial tyrocidine was added and tyrocidine was extracted with ethanol from the sedimentable material and separated from most other contaminating materials by passage through a column of acid alumina as described in the text. The ¹⁴C content of the eluents from these columns was determined and the amounts (μmol) of the various amino acids incorporated were calculated.

¹⁴ C-labelled amino acid	Amino acid incorporated (nmol/h per 15 mg of protein)	
	Before acid alumina	After acid alumina
L-Phenylalanine	15.7	13.0
DL-Phenylalanine	13.0	12.6
DL-Tryptophan	22.9	19.6
L-Tyrosine	11.4	9.3
DL-Valine	10.2	9.3
L-Glutamine	8.7	7.8
L-Leucine	9.3	9.0
Glycine	0	0
L-Serine	0.35	0.08

Fig. 7. Chromatography on a column of Sephadex LH-20 of material extracted from an incubation mixture containing L-[U- ^{14}C]phenylalanine as described in Table 2 and then eluted from an acid alumina column with ethanol

Conditions for chromatography and determination of the ^{14}C radioactivity (●) and the E_{280} (○) of the fractions were as indicated in Fig. 3 legend.

result was obtained with material derived from an incubation with L-[U- ^{14}C]glutamine. Although radioactivity was eluted slightly later than the commercial tyrocidine, it was possible that the amino acid composition of the commercial tyrocidine was different from that produced by *B. brevis* A.T.C.C. 10068 and that this difference was sufficient to allow the two species to be partially separated on the column.

Bartley (1969) had observed a similar behaviour of ^{14}C -labelled material that had been extracted from whole cells growing in AGM medium in the presence of [^{14}C]phenylalanine. For example, 100ml cultures were grown to a cell density of 0.65mg dry wt./ml (post-exponential phase) and a 25ml portion was transferred to a 100ml flask. After this portion had been shaken for 10min, 2.5 μCi of L-[U- ^{14}C]phenylalanine (0.62Ci/mol) was added and shaking was continued for a further 15min. Trichloroacetic acid was then added to give a final concentration of 5% (w/v), and material was extracted by a method similar to method A except that HCl was not added and the ethanol supernatants were evaporated to dryness. A small amount of gramicidin was added, to

Fig. 8. Chromatography on a column of Sephadex LH-20 of commercial tyrocidine and material extracted from whole *B. brevis* cells incubated with L-[U- ^{14}C]phenylalanine as described in the text

Commercial tyrocidine and the extracted material were chromatographed on a column (13cm $\times$ 2cm diam.) of Sephadex LH-20. Elution was with aq. 90% dimethylformamide, and 1ml fractions were collected. A 0.3ml portion of each fraction was taken for counting of radioactivity by solid-scintillation counting; the remainder was diluted with 2ml of solvent and its E_{280} value determined. ●, ^{14}C radioactivity; ○, E_{280} .

act as a column marker, and also 3mg of commercial tyrocidine. These were separated on a column (13cm $\times$ 2cm diam.) of Sephadex LH-20, and 0.3ml of each 1ml fraction was dried on to aluminium planchets and its radioactivity counted. Then 0.5ml of each fraction was diluted to 2.0ml with aq. 90% (v/v) dimethylformamide and its E_{280} value determined (Fig. 8). When allowances are made for the different column sizes it seems that the relationship between radioactivity and E_{280} value is similar to that

found previously (Fig. 7). It therefore seems likely that the material produced by cell extracts from cells growing in PYN medium is very similar to material produced by whole cells growing in AGM medium in the presence of phenylalanine.

Discussion

As a possible four out of the ten amino acid residues of tyrocidine can be tryptophan and three can be phenylalanine, probably the most sensitive method that can be used to study tyrocidine biosynthesis is to measure the incorporation of either [^{14}C]tryptophan or [^{14}C]phenylalanine into material with the properties of tyrocidine. Cells normally metabolize phenylalanine to other compounds less readily than they do tryptophan, and hence the incorporation of phenylalanine rather than tryptophan may be a more specific measure of the tyrocidine-biosynthetic process.

Cell extracts from cells grown with PYN medium incorporated [^{14}C]phenylalanine into material that was insoluble in acid, soluble in ethanol and behaved like tyrocidine and gramicidin when subjected to paper chromatography and paper electrophoresis done by methods similar to those of Uemura *et al.* (1963). Acid hydrolysis of the material followed by paper chromatography and radioautography showed only one radioactive spot, which corresponded to the position of phenylalanine. Consequently we considered that we were measuring the tyrocidine-biosynthetic process.

However, [^{14}C]proline incorporation into this material was much less than one-third of that found for [^{14}C]phenylalanine, and incorporation of [^{14}C]aspartic acid and [^{14}C]glutamic acid was negligible. Proline is a necessary constituent of tyrocidine, linked to the carboxyl group of a D-phenylalanine molecule and the amino group of either an L-phenylalanine or an L-tryptophan molecule. Incorporation of phenylalanine and proline might thus be expected to be concomitant. Although aspartic acid and glutamic acid are found in tyrocidine as asparagine and glutamine, the conditions in the extract with ATP and high endogenous ammonia should have led to the formation of [^{14}C]asparagine and [^{14}C]glutamine from [^{14}C]aspartic acid and [^{14}C]glutamic acid respectively. Consequently each of the alternative substrates for the tyrocidine asparagine and glutamine residues should have been labelled with ^{14}C .

It also seemed that the measured incorporation of phenylalanine was not a result of net peptide bond synthesis, for added ATP and/or an ATP-generating system was not essential for incorporation. An endogenous source of ATP was not detected. We concluded that either the measured incorporation of phenylalanine had been obtained by exchange into preformed peptide, in which case not all amino acid

residues could exchange, or the material that behaved like tyrothricin was not tyrothricin.

Extracts from cells grown with AGM medium also incorporated [^{14}C]phenylalanine and [^{14}C]tyrosine into material that was extracted with tyrothricin. Material formed from [^{14}C]phenylalanine behaved like tyrothricin on paper chromatography with a butan-1-ol-acetic acid-water solvent. The ^{14}C from this material was also eluted from a column of Sephadex LH-20 in those fractions that contained commercial tyrocidine, but the fractions that contained the most tyrocidine (E_{280}) were not those that contained the most ^{14}C (Fig. 3), and only 8% of the added radioactivity was recovered in the eluent from a column of acid alumina. As more than 97% of commercial tyrocidine and gramicidin was eluted from acid alumina, it was concluded that most of the [^{14}C]phenylalanine incorporated was not present in tyrothricin. When the ^{14}C -labelled material that was eluted from acid alumina was run through a Sephadex LH-20 column, the ^{14}C appeared in fractions that were so far away from those containing tyrocidine (Fig. 4) that it was concluded that none of the [^{14}C]phenylalanine originally incorporated was present in either gramicidin or tyrocidine. Obviously the ability of material to be eluted from acid alumina is not a sufficient criterion for establishing its identity with tyrocidine. This method is currently employed by Fujikawa *et al.* (1968a).

Experiments with material extracted from incubations with [^{14}C]tyrosine showed that on silica-gel thin layers some of the ^{14}C ran to the same position as gramicidin, but none to that of tyrocidine. ^{14}C was also present in those fractions from a Sephadex LH-20 column that also contained gramicidin (Fig. 5), and 14% of the added radioactivity was eluted from acid alumina. Therefore, although tyrocidine containing [^{14}C]tyrosine was not produced by the extracts, it was possible that some gramicidin was. However, if this had been so it would have been expected that some of the material extracted from incubations with [^{14}C]phenylalanine would behave like gramicidin (phenylalanine is a normal constituent of unfractionated gramicidin), and as this was not so it was concluded that gramicidin was not being produced. Unfortunately there was insufficient ^{14}C -labelled material from the [^{14}C]tyrosine incubation that was eluted from the acid alumina to be separated with the Sephadex LH-20 column. However, it is clear that the material produced from [^{14}C]tyrosine was different from that produced from [^{14}C]phenylalanine.

These experiments clearly demonstrate that none of the methods used to identify tyrocidine and gramicidin will individually satisfactorily establish their identities. The extraction procedures are certainly not specific for tyrothricin, and the paper-chromatographic and electrophoretic methods of

Uemura *et al.* (1963), although suitable for separating gramicidin from tyrocidine, do not separate these peptides from other unknown materials. The separation on silica-gel thin layers with the butan-1-ol-acetic acid-water solvent again separates gramicidin and tyrocidine and also separates tyrocidine from unknown materials. However, gramicidin is not completely separated from unknown materials, and amino acids such as phenylalanine and leucine run very close to the position of tyrocidine. The method used by Roskoski *et al.* (1970a) to establish the identity of tyrocidine involves precipitation with acid, extraction with butan-1-ol-chloroform and chromatography of ethanol-HCl-soluble materials on silica-gel thin layers. We have no reason to believe that this extraction procedure would adequately distinguish between tyrocidine and non-tyrocidine materials. Although this might not be so important when studying incorporation by partially purified enzyme systems, it would be essential to examine these methods in more detail if they were to be used for work with whole cells or with crude extracts.

The most effective means for purifying tyrothricin is with acid alumina, when amino acids and other contaminating materials are mostly adsorbed. In our experience the identity of the material eluted from acid alumina with tyrocidine and gramicidin can then be most easily established with the column of Sephadex LH-20 and dimethylformamide solvent. Special care has to be taken when the measured rate of incorporation of an amino acid is below 0.05 nmol/h per mg of protein, for until rates higher than this are achieved it seems that tyrocidine is not the predominant compound present in the extracted material.

The separation of gramicidin (mol.wt. 1882) from tyrocidine (mol.wt. 1308) achieved on the Sephadex LH-20 column was perhaps greater than expected for a process involving only gel filtration. In some solvents molecular-weight determinations indicate that gramicidin exists as a dimer [estimated mol.wt. up to 4426 (Sarges & Witkop, 1965)], and this difference would now account for the separation obtained and for the finding that gramicidin was eluted very quickly after dinitrophenyl-insulin. Sarges & Witkop (1965) found that gramicidin behaved like a monomer in dimethylformamide, so it would be necessary to postulate that the presence of 10% of water in dimethylformamide encourages dimerization. Alternatively the separation obtained might be due to tyrocidine being more strongly adsorbed on the Sephadex LH-20 than is gramicidin. When the amount of tyrocidine added to the column was increased the first fraction number to contain tyrocidine decreased. If there is a normal convex adsorption isotherm for the adsorption of tyrocidine by Sephadex LH-20, then as the concentration of peptide added to the column increases a larger

proportion of the tyrocidine will remain in solution, and it follows that the peptide peak should broaden and move toward a lower elution volume, but with the same trailing edge. This behaviour was observed (Fig. 2).

Sephadex G-25, which is the starting material for preparing Sephadex LH-20, does adsorb tyrocidine, and the strength of this adsorption in 10% acetic acid is increased as the number of tryptophan residues in the molecule increases (Ruttenberg *et al.*, 1965). There could be a similar effect with the Sephadex LH-20 and the dimethylformamide solvent. Materials derived from incubations with L-[U-¹⁴C]-phenylalanine or L-[U-¹⁴C]-glutamic acid and eluted from a column of acid alumina were eluted slightly later than commercial tyrocidine (Fig. 7). That this material was tyrocidine was clearly indicated by the finding that all those constituent amino acids of tyrocidine that were tested were also incorporated into this material (Table 2). There is therefore a possibility that tyrocidines with different amino acid compositions do adsorb differently on the Sephadex LH-20 in dimethylformamide solvent.

The ¹⁴C-labelled material that is different from tyrocidine but that is produced by extracts incubated with ¹⁴C-labelled amino acids is obviously heterogeneous. As the formation of these materials does not require ATP, and the incorporation in one case was increased by ribonuclease and was normal in the presence of either chloramphenicol or puromycin, it seems that the enzyme system responsible is different from that described by Uemura *et al.* (1963) and Okuda *et al.* (1964b) and that was claimed to produce tyrocidine. However, we made no attempts to modify conditions to try to improve incorporations into these materials, so it is still possible that such improvements might have revealed more properties that could relate it more clearly to the system described by the above workers and might explain why they assumed originally that the tyrocidine-biosynthetic process was similar to that involved in protein biosynthesis.

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