Experiments Relating to the Biosynthesis of Bacilysin

By JOAN ROSCOE* AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(Received 9 February 1966)

1. Bacilysin, a peptide which yields L-alanine and L-tyrosine on acid hydrolysis, was produced by a strain of *Bacillus subtilis* (A 14) in a chemically defined medium containing glucose, ammonium acetate or ammonium chloride, potassium phosphate and other inorganic salts, and ferric citrate. 2. Under the conditions used growth was diphasic. Bacilysin was formed during the second phase of slower growth, and there was little production during the stationary phase. Nevertheless, bacilysin production occurred when protein synthesis was inhibited by chloramphenicol. It thus appears that there is no obligatory coupling of protein synthesis and bacilysin synthesis. 3. When DL-[1-¹⁴C]alanine was added to a growing culture of *B. subtilis*, ¹⁴C was incorporated into bacilysin, which contains an N-terminal alanine residue. 4. Under similar conditions virtually no ¹⁴C was incorporated into bacilysin from DL-[2-¹⁴C]tyrosine, L-[U-¹⁴C]tyrosine or [1-¹⁴C]acetate, although these compounds were used by the cell for the biosynthesis of other substances. These results indicate that neither tyrosine nor acetate is a precursor of the fragment of bacilysin which yields tyrosine on hydrolysis with hot 6N-hydrochloric acid. 5. The tyrosine-yielding fragment of bacilysin was labelled with ¹⁴C from [1,6-ring-¹⁴C₂]shikimic acid. The biosynthesis of bacilysin thus appears to involve a diversion from the pathway leading to aromatic amino acids at the shikimic acid stage, or a subsequent one.

Bacilysin, an antibiotic produced by *Bacillus subtilis* which causes lysis of growing staphylococci, is a dipeptide containing an N-terminal L-alanine residue and a second residue which yields tyrosine on acid hydrolysis. The tyrosine-yielding moiety contains ketonic and epoxide groups and possible structures for this moiety have been suggested (Rogers, Newton & Abraham, 1965a; Rogers, Lomakina & Abraham, 1965b). The present work was designed to provide information about the relationship, if any, of protein synthesis to bacilysin production in *B. subtilis*, and about the precursors of the bacilysin molecule.

METHODS

Culture media. The concentrations of the components of the media are given in g./l. unless otherwise stated. In all cases, solutions of glucose were autoclaved separately from solutions containing the other constituents of the media. Media were made up in distilled water.

The following basal media were used, to which glucose and ferric citrate solutions and a solution of the 'oligodynamic' mixture of Pollock & Kramer (1958) were added in different amounts.

Basal medium A. This contained: KH₂PO₄, 6; K₂HPO₄, 14; KCl, 0.5; MgSO₄·7H₂O, 0.5; ammonium acetate, 3.43. Its pH was 7.0 without further adjustment. This medium consisted essentially of the phosphate buffer of Davis minimal medium (Davis & Mingioli, 1950) at double strength together with the salts (other than FeCl₃) of the medium of Rogers *et al.* (1965a).

Basal medium B. This was the same as basal medium A except that it contained NH₄Cl (2.39) instead of ammonium acetate.

Maintenance of *B. subtilis* A 14 (NCIB 9593). The organism was maintained as described by Rogers *et al.* (1965a).

Inocula. A fresh slope was streaked from one of the stored stock and incubated at 37° for 20–30 hr. In most cases a loopful was used to inoculate the following medium in a 500 ml. conical flask with four vertical indentations, each 1 cm. × 10 cm., containing: basal medium A, 100 ml.; Oxoid nutrient broth, 1.0 ml.; D-glucose, 4 ml. The flask was placed on a rotary shaker (160–170 rev./min.; 2 in. throw) at 32–34° for 15–18 hr. The supernatants showed no activity against *Staphylococcus aureus* on those occasions on which they were tested. The cells were separated from these cultures by centrifugation, resuspended in half the original volume of fresh basal medium, and again separated by centrifugation.

For some experiments basal medium B replaced basal medium A in the medium used for the growth of inocula.

Production of bacilysin. Cells from cultures grown to produce inocula were resuspended in the medium used for

* Present address: Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass., U.S.A.

production. Two media (1 and 2) were used. Medium 1 was made up as follows (ml./l.): basal medium A, 954; α -glucose, 40; oligodynamic mixture, 1; ferric citrate (20 mg./ml.), 5. The ferric citrate solution contained 20 mg./ml. each of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and trisodium citrate dihydrate. Medium 2 differed from medium 1 only in containing basal medium B in place of basal medium A. As far as possible the size of the inoculum was adjusted to give an initial extinction between 0.9 and 1.1 in a Spekker absorptiometer (Hilger and Watts Ltd., London, N.W. 1) with a neutral grey filter. This corresponds to a dry wt. of 0.41–0.45 mg./ml.

For preliminary experiments the cultures (10 ml.) were grown in L-tubes (inverted T-tubes). The tubes, of 40 ml. capacity, were rocked 50 times/min. in a water bath at 33–34°. In further experiments (unless stated otherwise) cultures (100 ml.) were grown at 32–34° in 500 ml. Erlenmeyer flasks, without indentations, shaken at 160–170 rev./min. When more than one flask was required for a single experiment the contents of all the flasks were rapidly mixed and redistributed in the hot room (37°) between the second and third hours of incubation. This was done to minimize variations between the flasks.

Estimations of bacterial growth. These were made by measurements of the opacity of cultures in L-tubes (1.7 cm. approx. diam.) with a Spekker absorptiometer. When necessary cultures were diluted with basal medium before extinctions were determined.

Assay of antibacterial activity. Bacilysin was assayed by the cylinder-plate method in a manner similar to that described by Rogers *et al.* (1965a). *S. aureus* (N.C.T.C. 6571) was the test organism and solutions containing 4, 8 and 16 units of cephalosporin C/ml. respectively were used as standards. The units of bacilysin/ml. assigned to a given sample were equal to the units of cephalosporin C/ml. required to give a zone of inhibition of the same diameter.

Before assay of bacilysin in culture supernatants containing chloramphenicol the latter was removed by two extractions with 1 vol. of ethyl acetate followed by two extractions with 1 vol. of ether.

Bioautographs. Agar plates (12 in. square) which had been seeded with *S. aureus* (N.C.T.C. 6571) were used. After paper chromatography or electrophoresis or both the paper was dried in air and rinsed three times in diethyl ether to remove traces of solvent. It was placed in contact with the agar for 30 min. and then removed. The plates were subsequently incubated at 37° overnight.

Estimation of bacterial protein. Samples (5 ml.) of culture were pipetted into 0.7 ml. of 40% (w/v) trichloroacetic acid. The mixtures were kept at 4° overnight and then heated for 15 min. in a boiling-water bath. After centrifugation the pellets were washed with an equal volume (usually about 5 ml.) of absolute ethanol and the mixture was again centrifuged. The resulting pellet was resuspended in a known volume (1–2.5 ml.) of N-NaOH and the suspension heated in a boiling-water bath for 10 min. After centrifugation to remove insoluble material (cell wall) the supernatant was diluted if necessary with N-NaOH before estimation of its protein content. Protein was estimated with the Folin-Ciocalteu reagent as described by Lowry, Rosebrough, Farr & Randall (1951). The reagent (British Drug Houses Ltd., Poole, Dorset) was diluted 1:1 with water. Bovine serum albumin (Armour Laboratories, Eastbourne, Sussex) was used as standard (25–125 $\mu\text{g.}$) and extinctions were read at 500 m μ .

Estimation of glucose. Estimations were made by the method of Huggett & Nixon (1957) with glucose oxidase and peroxidase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Intracellular amino acids and cell lipid. Bacterial cells were separated from 10 ml. of culture fluid by centrifugation and washed with 5 ml. of the medium used for growth. The pellet of cells was stirred with 1 ml. of 70% (v/v) ethanol. After centrifugation the supernatant was removed, diluted with water and freeze-dried to give a residue containing amino acids in the intracellular pool.

The pellet remaining after extraction with 70% (v/v) ethanol was extracted twice with 1 ml. of chloroform-methanol (2:1, v/v). Solvent was removed from the extract in a current of air to yield a residue containing cell lipid (Lascelles & Szilágyi, 1965).

Paper electrophoresis and chromatography. Electrophoresis at pH 1.8 (70 v/cm. for 1 hr.) followed by chromatography in the second dimension in butan-1-ol-acetic acid-water (4:1:4, by vol.) and electrophoresis at pH 4.5 (70 v/cm. for 30 min.) were carried out with Whatman no. 1 paper under conditions similar to those used by Rogers *et al.* (1965a). Spots were coloured with ninhydrin.

Radioactive compounds. [1,6-ring- $^{14}\text{C}_2$]Shikimic acid (10–15 mc/m-mole; 61 $\mu\text{C/mg.}$) was from Calbiochem, Lucerne, Switzerland. All the other radioactive compounds used were from The Radiochemical Centre, Amersham, Bucks. These were: DL-[1- ^{14}C]alanine (21.7 mc/m-mole; 245 $\mu\text{C/mg.}$); DL-[2- ^{14}C]tyrosine (15.7 mc/m-mole; 87 $\mu\text{C/mg.}$); L-[U- ^{14}C]tyrosine hydrochloride (6.2 mc/mole; 28.4 $\mu\text{C/mg.}$); sodium [1- ^{14}C]acetate (35.8 mc/m-mole; 437 $\mu\text{C/mg.}$).

Measurements of radioactivity. The radioactivities of spots on paper were determined with a Geiger-Müller detector with a thin mica end window mounted in a portable case. Pulses were counted on a linear decade scaler (model 1800; Isotope Development Ltd., Reading). The area of the paper to be counted was isolated with a mask of X-ray film of appropriate size. The counting efficiency under these conditions was about 2%. Samples of solutions of radioactive compounds which had been eluted from paper with water and solutions of cell protein were plated on lens tissue in aluminium planchets and counts made at infinite thinness with a gas-flow detector with a thin mica end window (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). This was used with a Nuclear-Chicago 8703 series scaler and C.110B sample changer. The counting efficiency was about 20%.

For the preparation of radioautographs after paper electrophoresis and chromatography the paper was placed in contact with Kodak Blue Brand X-ray film (14 in. $\times$ 17 in.). Films were developed after 21 days.

Preparation of bacilysin concentrates. To obtain samples of bacilysin which were suitable for analysis by paper electrophoresis and chromatography the following procedure was adopted. Bacterial cells were separated by centrifugation. Acetone was added to the crude supernatant fluid (9 to 10 ml.) to give a concentration of 70% (v/v). Whitish mucus was separated by centrifugation of the mixture at 3000g for 5 min. The supernatant fluid was concentrated *in vacuo* in a rotary evaporator to about 1 ml. The concentrate was removed from the flask and the latter rinsed with 0.5 ml. of water. Acetone was then added to the combined concentrate and washing to give a concentration of 70% (v/v). A further mucus pellet separated on

centrifugation and was washed with 1 ml. of 70% (v/v) acetone. The pooled supernatants (about 6 ml.) were concentrated to about 0.2 ml. in a rotary evaporator, the evaporation being carried out in a weighed tube. The yellow cloudy solution was centrifuged (3000g for 5 min.) and the clear yellowish supernatant transferred to a second weighed tube. Samples (about 25 μ l.) of this solution were used for paper electrophoresis and chromatography and further samples were appropriately diluted (1 in 20–1 in 140) and tested for antibacterial activity.

RESULTS

Production of bacilysin. The effect on bacilysin production of changes in some of the constituents of medium 1 (see the Methods section) was studied in several experiments. Omission of the oligodynamic solution (Pollock & Kramer, 1958), or an increase of its concentration 100-fold, did not produce any significant change in the amount of bacterial growth or in the amount of bacilysin formed. In contrast, omission of ferric citrate from the medium caused some reduction in growth and a reduction in the amount of bacilysin produced to about 40% of the maximum found with 100 mg. of ferric citrate/l. When the concentration of ferric citrate was increased to 250 mg./l. the production of bacilysin fell by about 50%. Bacilysin production was lower when the concentration of glucose in the medium was halved and was not increased when the glucose concentration was doubled. In medium 1 production began when about 50% of the glucose had been utilized.

Fig. 1. Growth of *B. subtilis* (A 14) in medium 1 and production of bacilysin. ●, Extinction of culture in Spekker absorptiometer; ○, bacilysin (units/ml.). No bacilysin was detected before 2.5 hr.

Under the conditions used, the highest yields of bacilysin were obtained in 500 ml. shake-flasks when the latter contained from 50 to 100 ml. of medium. When the efficiency of aeration was increased or decreased, by use of 25 ml. and 200 ml. of medium respectively, the yield (determined 7 hr. after inoculation) fell by approx. 50%. On the other hand, the amount of bacterial growth rose progressively as the volume of medium was decreased and with 25 ml./flask was more than twice that with 200 ml.

Fig. 1 shows the growth of *B. subtilis* A 14 and the production of bacilysin in medium 1 in a shake-flask under standard conditions (see the Methods section). There was some variation in the length of the lag phase from one experiment to another, but in each case growth was diphasic. Most of the bacilysin was produced during the slower growth in the second phase and there was little or no production during the subsequent stationary phase. The maximum titre (32 units/ml.) was of the same order as that obtained in earlier experiments with a different medium (Rogers *et al.* 1965a). In both cases virtually all the bacilysin was formed during a 2–3 hr. period.

In medium 2 the growth of the culture and production of bacilysin followed courses similar to those shown in medium 1. However, the pH of the culture fluid fell to 6.5 during growth in medium 2, whereas it did not fall below 6.7 in medium 1. No clumping of the bacterial cells occurred in medium 2, whereas some did occur in medium 1.

Effect of chloramphenicol on bacilysin and protein synthesis. Fig. 2 shows the effect of chloramphenicol (final concn. 50 μ g./ml.) on protein synthesis and bacilysin production by *B. subtilis* grown in shake-flasks in medium 1. The chloramphenicol was added to one flask when rapid production of bacilysin was about to begin and to two other flasks 1 hr. and 1.75 hr. later respectively. Protein estimations at the times of addition and subsequent estimations 8 hr. after inoculation indicated that protein synthesis was completely inhibited by chloramphenicol in the concentration used. In contrast, bacilysin continued to be produced after the addition of chloramphenicol, though the amount which had been formed 8 hr. after inoculation was in each case less than that in the control culture. The earlier the chloramphenicol was added, the lower the yield of bacilysin.

Incorporation of radioactivity from ^{14}C -labelled compounds into bacilysin

Determinations were made of the radioactivity of bacilysin produced in cultures of *B. subtilis* A 14 to which ^{14}C -labelled compounds had been added.

For this purpose the bacilysin in concentrates of the culture supernatants (see the Methods section) was resolved from other substances by electrophoresis and chromatography on paper. The amounts of bacilysin in the concentrates were determined by biological assay.

After electrophoresis at pH 1.8, followed by chromatography in the second dimension, between seven and ten ninhydrin spots were revealed by a ninhydrin spray. A typical distribution of ninhydrin-positive spots is shown in Fig. 3. Bacilysin was represented by spot 1, since this spot increased in intensity when a highly purified sample of bacilysin (Rogers *et al.* 1965a) was added to the concentrate and a zone of inhibition corresponded approximately in position to that expected for spot 1 was obtained when electrophoresis and chromatography was followed by bioautography. Spot 6 corresponded in position to alanine and spot 3 to tyrosine.

Incorporation of ^{14}C from DL-[1- ^{14}C]alanine. A culture of *B. subtilis* A 14 was grown in a shake-flask in medium 1. After 6 hr., when the change in the rate of growth (Fig. 1) indicated that the

production of bacilysin was under way, a sample of the culture was taken for antibacterial assay and 10 ml. portions were added to each of four tubes, two of which contained $10\mu\text{C}$ of DL-[1- ^{14}C]alanine in 0.1 ml. of water. After incubation for 1 hr. the contents of the tubes containing no radioactivity were used for determinations of growth and antibacterial assay. A concentrate was prepared from one of the tubes to which [1- ^{14}C]alanine had been added. A sample of the latter was assayed for bacilysin after suitable dilution and two further samples were subjected to electrophoresis and chromatography on paper. One of the papers was sprayed with ninhydrin and the spot corresponding with bacilysin was found to be highly radioactive (976 counts/min.). A radioautograph, prepared from the second paper, showed no other spot with comparable radioactivity (Table 1). The specific radioactivity of the bacilysin formed in the presence of [1- ^{14}C]alanine, relative to that found in experiments with other ^{14}C -labelled compounds, is shown in Table 2.

Failure to demonstrate incorporation of radioactivity from [^{14}C]tyrosine. An experiment with DL-[2- ^{14}C]tyrosine ($10\mu\text{C}$) was carried out under conditions which were essentially the same as those with [^{14}C]alanine. A similar experiment was carried out subsequently with L-[U- ^{14}C]tyrosine ($10\mu\text{C}$) in medium 2. In neither case was more than a trace of radioactivity found in the spot

Fig. 2. Effect of chloramphenicol on bacilysin production and protein synthesis in *B. subtilis* (A 14). $\circ$ — $\circ$, Bacilysin produced by control culture; $\circ$ — $\circ$, bacilysin after addition of chloramphenicol; $\bullet$ — $\bullet$, protein synthesis in control culture; $\bullet$ — $\bullet$, protein after addition of chloramphenicol. The arrows denote the times after inoculation at which chloramphenicol was added. Values for bacilysin given as zero signify that the amount was less than 1 unit/ml.

Fig. 3. Behaviour of compounds present in a concentrate of the culture fluid of *B. subtilis* (A 14). Electrophoresis on paper (70 v/cm. for 1 hr.) was followed by chromatography in the second dimension. The density of shading indicates the relative intensities of spots revealed by ninhydrin. 1, Bacilysin; 3, tyrosine; 6, alanine. The compounds responsible for the remaining spots have not been characterized. Spot 7 showed no colour with ninhydrin but was labelled by ^{14}C from [1- ^{14}C]alanine (Table 1).

corresponding to bacilysin after paper electrophoresis (Table 2). In the two experiments the spots corresponding to tyrosine were strongly radioactive (11 000 and 2070 counts/min. respectively).

To determine whether added [^{14}C]tyrosine had entered the bacterial cell, solutions of protein fractions were prepared as described in the Methods section and compared with a similar fraction prepared from the culture to which [^{14}C]alanine had been added. Protein was labelled with ^{14}C from DL-[2- ^{14}C]tyrosine and its specific radioactivity (about 200 $\mu\text{C}/\text{mg.}$) was similar to that labelled from DL-[1- ^{14}C]alanine. Protein labelled from L-[U- ^{14}C]tyrosine showed a higher specific radioactivity (978 $\mu\text{C}/\text{mg.}$).

Incorporation of [1- ^{14}C]acetate. A preliminary

experiment with [1- ^{14}C]acetate in medium 1 failed to show a significant loss of radioactivity from the medium during growth of the culture. It seemed possible that this was due to the large dilution (> 100-fold) of radioactive acetate by the unlabelled acetate in the medium, and to the utilization by the cells of no more than a very small proportion of the total acetate present. An experiment to determine whether ^{14}C from [1- ^{14}C]acetate was incorporated into bacilysin was therefore carried out in medium 2, in which the ammonium acetate of medium 1 was replaced by ammonium chloride.

The conditions used were similar to those with DL-[1- ^{14}C]alanine, except that 10 ml. of a culture of *B. subtilis* which had grown for 6 hr. in medium 2 was incubated for 1.5 hr. with 25 μC of sodium [1- ^{14}C]acetate. A sample of the bacilysin concentrate was assayed and a further sample subjected to electrophoresis and chromatography on paper. The results are given in Table 2. They show that there was virtually no labelling of the bacilysin.

To determine whether added [1- ^{14}C]acetate was utilized for the biosynthesis of other substances in *B. subtilis* A 14, the lipid fraction and material from the intracellular pool (prepared as described in the Methods section) were examined. The lipid fraction was dissolved in 200 $\mu\text{l.}$ of chloroform-methanol (2:1, v/v) and samples (31.6 $\mu\text{l.}$) of the solution were counted on planchets. They showed approx. 1000 counts/min. The material from the intracellular pool was dissolved in water (200 $\mu\text{l.}$) and 25 $\mu\text{l.}$ samples of the solution were subjected to chromatography in butan-1-ol-acetic acid-water and to electrophoresis at pH 4.5 on paper. The major

Table 1. *Incorporation of ^{14}C from DL-[1- ^{14}C]alanine into bacilysin and other extracellular compounds*

Radioactive spots (revealed by radioautography) were counted on paper after electrophoresis and chromatography of a bacilysin concentrate (for details see the Methods section and the text). The spots are numbered as in Fig. 3. Radioactivity was not detected in spots 3, 4, 5 and 9.

Spot no.	Radioactivity (counts/min.)
1 (bacilysin)	934
2	97
6 (alanine)	10
7	105
8	68

Table 2. *Incorporation of ^{14}C from radioactive compounds into bacilysin*

Amounts of bacilysin given in the Table are those in samples of concentrates subjected to electrophoresis and chromatography and derived from about 1 ml. portions of culture supernatants. The amount of radioactivity added to the culture was 1 $\mu\text{C}/\text{ml.}$ in each case. For the calculation of specific radioactivities in $\mu\text{C}/\mu\text{mole}$ it was assumed that bacilysin had an antibacterial activity of 200 units/mg. and mol. wt. 270 (Rogers *et al.* 1965b) and that the efficiency of counting on paper was 2% so that 44 000 counts/min. represented 1 μC .

Radioactive compound added	Sp. radio- activity ($\mu\text{C}/\mu\text{mole}$)	Total bacilysin (μmole)	Bacilysin formed after addition of ^{14}C -labelled compounds		Radioactivity of bacilysin formed		Approx. sp. radioactivity of bacilysin formed ($\mu\text{C}/\mu\text{mole}$)
					(counts/ min./ μmole)		
			(%)	(μmole)	(counts/ min.)	(counts/ min./ μmole)	
DL-[1- ^{14}C]Alanine	21.7	0.149	32	0.048	934	19450	0.44
DL-[2- ^{14}C]Tyrosine	15.7	0.097	49	0.047	6	128	< 0.003
L-[U- ^{14}C]Tyrosine	6.2	0.109	72	0.079	1	13	< 0.0003
[1,6- <i>ring</i> - $^{14}\text{C}_2$]Shikimic acid	10.15*	0.254	74	0.185*	803	4320	0.1
[1- ^{14}C]Acetate	35.8	0.122	72	0.088	5	57	< 0.001

* These values indicate that the molar quantity of shikimic acid added was less than that of the bacilysin subsequently formed.

ninhydrin-positive spot, which corresponded with glutamic acid, was radioactive (1744 counts/min.). An approximate estimate of the amount of glutamic acid present on the paper after electrophoresis (16.9 $\mu\text{g.}$) was made from its ninhydrin colour density in the manner described by Swallow & Abraham (1958). If bacilysin had been labelled with the same efficiency as intracellular glutamic acid from [^{14}C]acetate the bacilysin spot (23.9 $\mu\text{g.}$) should have shown about 1300 counts/min. on paper. The value found (Table 2) was 6 counts/min.

Incorporation of ^{14}C from [^{14}C]shikimic acid. The procedure followed in this experiment was similar to that used with L-[U- ^{14}C]tyrosine. After growth of *B. subtilis* for 5.75 hr. in medium 2, 10 ml. of culture was added to 10 μC of [1,6-ring- $^{14}\text{C}_2$]shikimic acid in a 1-tube and incubation continued for 1.5 hr. A sample of a bacilysin concentrate, prepared as described in the Methods section, was subjected to electrophoresis on paper at pH 1.8 followed by chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.) in the second dimension. Radioautography revealed one strongly radioactive spot (803 counts/min.), which was ninhydrin-positive and corresponded in position to bacilysin, among the compounds which had migrated towards the cathode. Significant, but less, radioactivity (182 counts/min.) was found in a position corresponding to tyrosine. Minor radioactive spots were present which were due to compounds whose R_F values were close to that of bacilysin, but which migrated slightly further and slightly less far respectively than bacilysin towards the cathode. One major and five minor radioactive spots were due to compounds which behaved as though they had no net charge at pH 1.8. The major spot, whose R_F value was slightly greater than that of bacilysin, appeared to be unchanged shikimic acid. Since this spot showed 28 800 counts/min., only about 35% of the added shikimic acid had been removed from the culture fluid during the experiment. Table 2 shows that the amount of radioactivity incorporated into bacilysin from 10 μC of [^{14}C]shikimic acid was 22% of that incorporated from 10 μC of DL-[1- ^{14}C]alanine.

The remainder of the crude labelled bacilysin from this experiment was applied to paper in five spots side by side and subjected to electrophoresis (70 v/cm.) at pH 1.8 for 1 hr. The position of the bacilysin from one of these spots was located by counting radioactivity on the paper and coloration with ninhydrin. The corresponding area of paper in line with the remaining spots was cut out and the bacilysin eluted with water. The material in the eluate was hydrolysed with 6N-hydrochloric acid at 105° for 18 hr., and the hydrochloric acid then removed *in vacuo* over phosphorus pentoxide and sodium hydroxide. The residue was dissolved

in water (50 $\mu\text{l.}$), and a sample (5 $\mu\text{l.}$) of this solution mixed with unlabelled tyrosine (8 $\mu\text{g.}$), and the mixture subjected to electrophoresis on paper at pH 1.8. Electrophoresis was carried out in parallel with a mixture of L-alanine, L-tyrosine and DL-phenylalanine (each 10 $\mu\text{g.}$) and with a hydrolysate of a highly purified preparation of unlabelled bacilysin (Rogers *et al.* 1965a).

The major radioactive spot from the hydrolysate of the labelled bacilysin coincided with the spot due to tyrosine, which was revealed by the ninhydrin and Pauli reagents. Almost all the remaining radioactivity (about 40% of the whole) was present in a compound which had migrated 0.86 times as far as tyrosine towards the cathode and which showed positive Pauli and ninhydrin reactions. The latter compound, together with tyrosine and alanine, was also detected by coloration with ninhydrin in the parallel hydrolysate of unlabelled purified bacilysin. Chromatography of a sample of the hydrolysate of the labelled bacilysin in butan-1-ol-acetic acid-water gave a radioactive spot corresponding to tyrosine (R_F 0.36) and a second spot (containing about 76% of the radioactivity found in tyrosine) with R_F 0.45. The second spot was shown by two-dimensional electrophoresis and chromatography to be due to the compound which had been found to migrate 0.86 times as far as tyrosine when subjected to electrophoresis alone. A similar spot was revealed on two-dimensional resolution of a hydrolysate of highly purified unlabelled bacilysin.

A protein fraction was prepared as described in the Methods section from the pellet of cells obtained, by centrifugation, from the culture to which [^{14}C]shikimic acid had been added. This fraction contained approximately 3.5 mg. of protein with a specific radioactivity of approximately 123 m μC /mg. Hydrolysis of a small sample of the protein with 6N-hydrochloric acid at 105°, followed by electrophoresis at pH 1.8, indicated that most of the radioactivity was in tyrosine and phenylalanine residues.

DISCUSSION

For the experiments carried out in the present work the culture media that were used had several advantages over the chemically defined medium of Rogers *et al.* (1965a). The addition of iron in the form of ferric citrate, instead of ferric chloride, gave media which were clear instead of turbid. The use of relatively high concentrations of phosphate buffer resulted in smaller variation in pH during growth. There was less tendency for the cells to clump in medium 1 than in that of Rogers *et al.* (1965a) and no clumping occurred in medium 2, in which the ammonium acetate of medium 1

had been replaced by ammonium chloride. The addition to media of an 'oligodynamic' solution (Pollock & Kramer, 1958) did not increase the yield of bacilysin in the present experiments, but the use of this solution was continued, since in earlier work it appeared necessary for maximum yields in some cases (H. J. Rogers, unpublished work).

Although the biosynthesis of bacilysin took place during cell growth, only a relatively small proportion of the antibiotic was formed during the early phase of the culture when growth was most rapid. Since bacilysin formation continued when protein synthesis was inhibited by chloramphenicol, there was no obligatory coupling between the biosynthesis of bacilysin and of protein. Most of the reported studies on the biosynthesis of peptide antibiotics have led to the conclusion that the formation of these substances involves mechanisms which are quite different from those of protein synthesis (for references see Abraham, Newton & Warren, 1965), although there is some dissent from this view (Okuda, Uemura, Bodley & Winnick, 1964a,b).

The addition of chloramphenicol to a culture of *B. subtilis* 3-25 hr. after inoculation, when rapid synthesis of bacilysin was about to begin, lowered the amount of bacilysin formed by 8 hr. to about 27% of that found in the control culture. When chloramphenicol was added later, during the phase of rapid production of bacilysin, the decrease in the rate of synthesis of the latter was considerably less. This is presumably due to an increase, during the growth of the culture, in the amounts of enzymes which are concerned, directly or indirectly, with bacilysin synthesis. When chloramphenicol is added to the culture enzyme synthesis is terminated and the subsequent rate of bacilysin production will depend on the amounts of the necessary enzymes which have already been formed.

Electrophoresis and chromatography on paper indicated that bacilysin was a major component of the mixture of compounds secreted by *B. subtilis* A 14 that were ninhydrin-positive and soluble in 70% (v/v) acetone. Of these compounds bacilysin (which contains an *N*-terminal alanine residue) was the one most heavily labelled by ^{14}C from [^{14}C]-alanine. The procedures used for determinations of the amount and radioactivity of the antibiotic formed in these small-scale experiments did not allow an accurate estimate to be made of the dilution of radioactivity which accompanied the incorporation of ^{14}C into bacilysin. But Table 2 indicates that with DL-[^{14}C]-alanine an approximate value for this dilution was 1:48.

Bacilysin yields L-tyrosine on acid hydrolysis, but DL-[^{14}C]-tyrosine did not function as a precursor of the antibiotic when added to a growing culture of *B. subtilis* A 14, despite the fact that ^{14}C

from DL-tyrosine was incorporated into cell protein with an efficiency similar to that of ^{14}C from DL-alanine. [^{14}C]-Acetate also failed to function as a precursor, although it was utilized for the synthesis of cell lipid and intracellular glutamic acid under the conditions employed.

In contrast, ^{14}C from [^{14}C]-shikimic acid was incorporated into bacilysin with a dilution of about 1:107, which is of the same order as, though higher than, that found with [^{14}C]-alanine. The higher values with shikimic acid can be partly accounted for by the fact that the amount of shikimic acid taken up by the bacterial cells was only 19% of the bacilysin formed on a molar basis, whereas virtually all the added [^{14}C]-alanine was taken up under similar conditions. However, various unknown factors, such as the pool sizes, rates of biosynthesis and utilization for other purposes of shikimic acid and alanine respectively are also involved. After acid hydrolysis of the labelled bacilysin most of the ^{14}C from shikimic acid was found in tyrosine and a smaller amount in an uncharacterized product which showed a positive Pauli reaction. These results indicate that neither tyrosine nor acetate is an intermediate in the biosynthesis of the fragment of bacilysin that yields tyrosine on acid hydrolysis, but that the formation of the latter involves a diversion from the biosynthetic pathway leading from glucose, via shikimic acid, to aromatic amino acids. Several compounds, including 3-enolpyruvoylshikimate 5-phosphate (Levin & Sprinson, 1964), chorismate (Gibson & Gibson, 1964) and prephenate (Weiss, Gilvarg, Mingioli & Davis, 1954), have been characterized as intermediates between shikimate and phenylalanine and tyrosine (Bohm, 1965). Whether all, or any, of these compounds are precursors of the tyrosine-yielding moiety of bacilysin remains to be determined.

We are indebted to the Medical Research Council for a grant for equipment and for a personal grant to J. R.

REFERENCES

- Abraham, E. P., Newton, G. G. F. & Warren, S. C. (1965). In *Biogenesis of Antibiotic Substances*, chapter 14, p. 169. Ed. by Vanek, Z. & Hostálek, Z. Prague: Publishing House of the Czechoslovak Academy of Sciences.
- Bohm, B. A. (1965). *Chem. Rev.* **65**, 435.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Gibson, M. I. & Gibson, F. (1964). *Biochem. J.* **90**, 248.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
- Lascelles, J. & Szilágyi, J. F. (1965). *J. gen. Microbiol.* **38**, 55.
- Levin, J. G. & Sprinson, D. B. (1964). *J. biol. Chem.* **239**, 1142.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Okuda, K., Uemura, I., Bodley, J. W. & Winnick, T. (1964a). *Biochemistry*, **3**, 100.
- Okuda, K., Uemura, I., Bodley, J. W. & Winnick, T. (1964b). *Biochemistry*, **3**, 108.
- Pollock, M. R. & Kramer, R. (1958). *Biochem. J.* **70**, 668.
- Rogers, H. J., Newton, G. G. F. & Abraham, E. P. (1965a). *Biochem. J.* **97**, 573.
- Rogers, H. J., Lomakina, N. & Abraham, E. P. (1965b). *Biochem. J.* **97**, 579.
- Swallow, D. L. & Abraham, E. P. (1958). *Biochem. J.* **70**, 364.
- Weiss, U., Gilvarg, C., Mingioli, E. S. & Davis, B. D. (1954). *Science*, **119**, 774.