Biosynthesis of Polymyxin E

BY

SADAAKI KOMURA

Institute for Protein Research
OSAKA UNIVERSITY

June, 1980

CONTENTS

INTRODUCTION

Peptide Antibiotics : A large number of peptide antibiotics have been discoverd and described in the literature in the past 40 years. These compounds have several distinctive features in their chemical structures. Most peptide antibiotics are in general much smaller in their size than proteins. Peptide antibiotics usually contain both L- and D-amino acid residues and sometimes L-and D-isomers of the same amino acid are present in a molecule. Some peptide antibiotics contain unusual amino acids such as N-methylated amino acid, β-amino acids, imino acids, and dihydroxyamino acids. Certain amino acids commonly found in proteins such as arginine, histidine and methionine are rarely found in peptide antibiotics. Some peptide antibiotics contain fatty acids, pyrimidines, amino sugars, hydroxy acids, and chromophores. Many peptide antibiotics possess cyclic structures, thus no free N- or C-terminals are found. If not cyclic, their N-terminal and C-terminal amino acid residues are often modified. Thus, it is a challenging problem to elucidate the biosynthetic mechanism of each of these diversified peptides.

Biosynthesis of peptide antibiotics: Biogenetic studies of peptide antibiotic in producer organisms indicated a biosynthetic mechanism different from that of proteins, because puromycin, chloramphenicol, actinomycin D, and other inhibitors of protein synthesis did not inhibit the peptide production. These findings incited studies of the mechanism of this nonribosomal peptide synthesis by cell-free enzyme systems. By the present time, several laboratories have reported cell-free systems of the biosynthesis of peptide antibiotics such as gramicidin S, tyrocid= ines, bacitracins, gramicidin A, edeine and malformin. Isolation of the multienzyme systems which catalyze peptide formation was achieved, and the following features of the biosynthetic mechanism were revealed.

(1) Activated amino acids are bound to the multienzyme complex as thioes=

ters on specific thiol groups. (2) The sequence of amino acids in peptides is determined by the location of these thiol groups on the multienzyme. (3) Elongation of the peptides starts from the N-terminal to the C-terminal end. (4) Growing peptides are also linked to the enzyme as thioesters, and an enzyme-bound phosphopantetheine participates in the peptidation reactions.

These studies were reviewed by Kurahashi (1), Perlman and Bodanszky (2), Lipmann (3) and Laland and Zimmer (4), and such a mechanism of peptide synthesis without the involvement of ribosomes or nucleic acids has been termed the "protein template mechanism", the "protein thiotemplate mechanism" or the "multienzyme thiotemplate mechanism". I consider the last term expresses best the features.

Polymyxins: In 1947, Stansly et al. (5) described the production, isolation and purification of an active principle from Bacillus polymyxa under the name of "polymyxin". Ainsworth et al.(6) and Benedict and Langlykke (7) independently reported an antibiotic "aerosporin", which appeared to be similar to polymyxin in its antibacterial spectrum. It became apparent that both were dealing with a family of chemically and biologicaly closely related polypeptides (8). Comparative chemical and biological studies were undertaken co-operatively between the groups, and it was agreed to use the generic term "polymyxin" for these antibiotics at a symposium organized by the New York Academy of Science in 1948 (9).

---

(1) Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445
(2) Perlman, D. & Bodanszky, M. (1971) Ann. Rev. Biochem. 40, 449
(3) Lipmann, F. et al. (1972) Adv. in Enzymology, 35, 1
(4) Laland, S. G. & Zimmer, T. L. (1973) in Essays in Biochemistry Vol. 9, pp. 31
(5) Stansly, P. G. et al. (1947) Bull. Johns Hopkins Hosp. 81, 43
(6) Ainsworth, G. C. et al. (1947) Nature, 160, 263
(7) Benedict, R. G. & Langlykke, A. F. (1947) J. Bact. 54, 24
(8) Jones, T. S. G. (1948) Biochem. J. 43, 26
(9) Symposium 1948, Ann. N. Y. Acad. Sci. 51, 855 (1949)

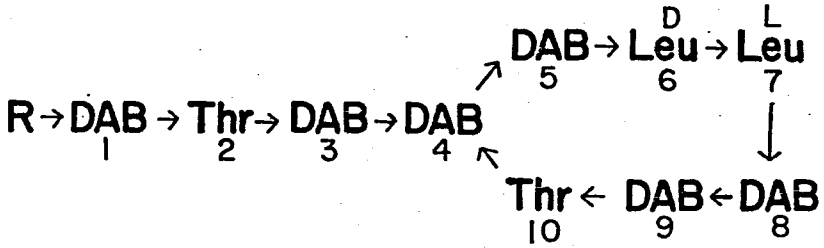
Alphabetical suffixes were adopted for different types which differed in their amino acid compositions. Aerosporin thus became polymyxin A and polymyxin was called polymyxin D.

Jones (10) isolated and described polymyxin E, while Koyama et al. (11) reported the isolation of an antibiotic from the culture fluid of a new species named B. polymyxa var colistinus. The latter was called colistin. Chemical analysis showed that colistin was a cyclic basic peptide which, on hydrolysis, gave 2,4-diaminobutyric acid, leucine, threonine and 6-methyloctanoic acid, indicating that it belongs to the polymyxin series of antibiotics (12-15). Polymyxin E and colistin were fractionated into two components, which were named polymyxin E_1 & E_2 and colistin A & B, respectively (16-18). Suzuki et al. elucidated the chemical structure of colistin A and B (16, 19-21), and further showed that colistin A and B were identical with polymyxin E_1 and E_2, respectively. Recently, polymyxin E_3 was reported by Withander and Heding (22).

---

(10) Jones, T. S. G. (1949) Ann. N. Y. Acad. Sci. 51, 909
(11) Koyama, Y. et al. (1950) J. Antibiotics Ser. B., 3, 457
(12) Kurihara, T. & Suzuki, K. (1953) J. Pharm. Soc. Japan 73, 414
(13) Oda, T. et al. (1953) J. Pharm. Soc. Japan 74, 1243
(14) Oda, T. and Ueda, F. (1954) J. Pharm. Soc. Japan 74, ,246
(15) Shoji, J. et al. (1959) J. Antibiotics Ser. B., 12, 365
(16) Suzuki, T. et al. (1963) J. Biochem. 54, 173
(17) Wilkinson, S. & Lowe, L. A. (1964) J. Chem. Soc. 4107
(18) Suzuki, T. et al. (1965) J. Biochem. 57, 226
(19) Suzuki, T. et al. (1963) J. Biochem. 54, 25
(20) Suzuki, T. et al. (1964) J. Biochem. 54, 412
(21) Suzuki, T. et al. (1964) J. Biochem. 56, 182
(22) Withander, L. & Heding, H. (1976) J. Antibiotics 29, 774

Compared with other peptide antibiotics of which biosynthetic mechanism has been studied, polymyxins have many unique structural features. They contain a number of residues of an unusual amino acid, 2,4-diaminobutyric acid, and fatty acid moiety linked by α-amide linkage to the N-terminal amino acid. They have a cyclic structure with a branched chain. It is hoped that to clarify the biosynthetic mechanism of polymyxin E would add new knowledge to the general understanding of peptide biosynthesis.



Polymyxin E_1: R=6-methylocatnoic acid (MOA)

Polymyxin E_2: R=isooctanoic acid (IOA)

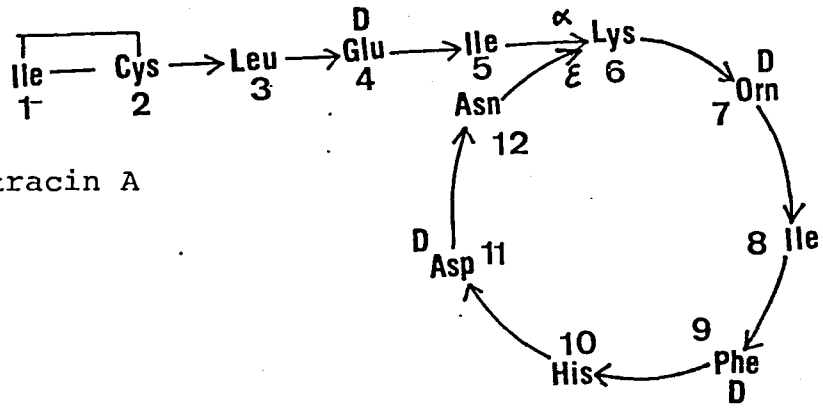
Polymyxin E_3: R=octanoic acid (OA)

DAB: 2,4-diaminobutyric acid

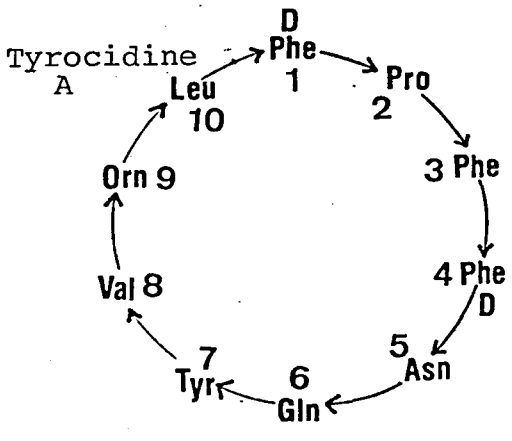
Structure of polymyxin E

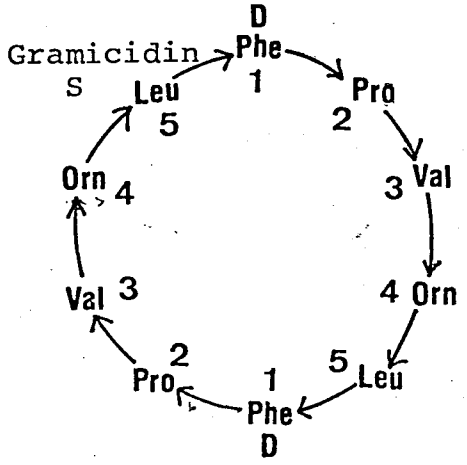
HCO Val Gly Ala Leu Ala Val Val Val Trp Leu Trp Leu Trp Leu Trp $NHCH_2CH_2OH$
1 5 10 15

Gramicidin A



Bacitracin A





PART I

Partial Purification and Properties of L-2,4-Diaminobutyric Acid Activating Enzyme from a Polymyxin E Producing Organism

---

Abbreviations; DAB, 2,4-diaminobutyric aicd; MOA, 6-methyloctanoic acid; IOA, isooctanoic acid; TCA, trichloroacetic acid.

SUMMARY

An L-2,4-diaminobutyric acid activating enzyme was found in crude extracts of Aerobacillus polyaerogenes, which produces polymyxin E_1 and E_2. The enzyme was partially purified by sonication of the cells, followed by ultracentrifugation, ammonium sulfate fractionation and DEAE-cellulose column chromatography. In addition to L-2,4-diamino= butyric acid, the enzyme activated L-leucine and L-threonine, which are constituent amino acids of polymyxin E. All three amino acids were bound to the enzyme as thioesters. These results suggest that polymyxin is synthesized by a multienzyme thiotemplate mechanism, in the same way as gramicidin S, tyrocidines, bacitracins and gramicidin A.

It is well-established that antibiotic peptides such as gramicidin S, tyrocidines, bacitracins and gramicidin A are synthesized by a multienzyme thiotemplate mechanism that involves neither RNA nor ribosomes (1-6). The polymyxin series of antibiotics contains a number of 2,4-diaminobutyric acid (DAB) residues and a fatty acyl moiety linked by an α-amide linkage to the N-terminal DAB residue, as shown in Diagram I. A number of biogenetic studies have indicated

Diagram I

that the synthesis of polymyxins is not inhibited by inhibitors of protein synthesis such as chloramphenicol, brorrelidin and puromycin (7-9), suggesting that the biosynthetic mechanism may be similar to that of gramicidin S and tyrocidines. However, very few in vitro studies on the biosynthesis of polymyxins have been carried out and the biosynthetic mechanism remains obscure.

Ciferri et al. (10), Brenner et al. (11), and Jayaraman et al. (12) reported the presence of an enzyme in cell-free extracts of polymyxin B producing bacteria (Bacillus polymyxa strain Pfizer 2459) that catalyzed an ATP-PP_i exchange reaction dependent on L-DAB. This enzyme activity was not found in mutant strains of B. polymyxa that had lost the ability to form polymyxin B (12). Ito et al. (9) failed to detect L-DAB activating activity in colistin (polymyxin E) producing organisms, but they reported the incorporation of L-[^{14}C]threonine into colistins by a cell-free enzyme system (13). The identity of colistins with polymyxin E was elucidated by Suzuki et al. (14). In this communication

I report the partial purification of an L-DAB activating enzyme from another strain of polymyxin E producing bacteria, Aerobacillus polyaerogenes. The enzyme was also found to activate L-leucine and L-threonine and to bind them as thioester.

MATERIALS AND METHODS

Chemicals and Enzymes — The following chemicals were obtained commercially: Sephadex G-50 (fine) from Pharmacia, Bio-gel P-300 from Bio Rad Laboratories, DEAE-cellulose (0.6 meq/g) from Serva Co., L-2,4-diaminobutyric acid and L-ornithine from Mann Research Laboratories, γ-amino-n-butyric acid from Wako Pure Chemical Industries Ltd., L-α-amino-n-butyric acid and dithiothreitol from Nakarai Chemicals Ltd., other amino acids from Tanabe Amino Acid Research Foundation, ATP from Sigma Chemical Co., DL-[4-^{14}C]2,4-diaminobutyric acid from Service Molécules Marquées Farbrique par CEA-France, L-[U-^{14}C]leucine (240 mc$_i$/mmole) and L-[U-^{14}C]threonine (70 mC$_i$/mmole) from Daiichi Pure Chemical Co., and [2,8-^{3}H]ATP (33.1 C$_i$/mmole) from New England Nuclear. ^{32}P-Labeled inorganic pyrophosphate was prepared according to the method of Nishikawa et al. (15). Inorganic pyrophosphatase [EC 3.6.1.1] from yeast was purchased from Boehringer Mannheim. N$^{\alpha}$-6-methyloctanoyl-2,4-diaminobutyric acid (MOA-DAB) and N$^{\alpha}$-isooctanoyl-2,4-diaminobutyric acid (IOA-DAB) were generous gifts from Dr. T. Suzuki.

Bacterial Strains and Growth Media — Polymyxin E_1 and E_2 producing Aerobacillus polyaerogenes, which had been isolated by Dr. K. Hayashi of Kyoto University from soils in Ikeda City, Osaka, was used in this study. Escherichia coli NIH J, used as a test strain for bioassay of polymyxin E, was kindly supplied by Dr. Y. Kimura of Mukogawa Women's University. The composition of agar slants on which A. polyaerogenes was maintained was as follows: glucose, 1.5 %; yeast extract, 0.5 %; ammonium sulfate, 2.0 %; K_2HPO_4, 0.26 %; $MgSO_4 \cdot 7H_2O$, 0.05 %; NaCl, 0.005 %; $FeSO_4 \cdot 7H_2O$, 0.001 %; agar, 1.5 %; the pH was adjusted to 7.2 with NaOH. The composition of a soluble starch medium for liquid culture was as follows : polypeptone, 1 %; meat extract, 0.05 %; $CaCO_3$,

1 %; KH_2PO_4, 0.1 %; soluble starch, 5 %; $MgSO_4 \cdot 7H_2O$, 0.01 %; $CuSO_4 \cdot 4H_2O$, 0.0005 %; the pH was adjusted to 7.2 with NaOH.

Cultivation of Bacteria — A loopful of cells of A. polyaerogenes grown on a slant was inoculated into 50 ml of the soluble starch medium and incubated at 28° for 16 h in a New Brunswick rotary incubator-shaker. An aliquot (10 ml) of the starter culture was transferred into a 2-liter flask containing 500 ml of the soluble starch medium. The cells were grown at 28° with vigorous shaking and harvested at the late logarithmic phase of growth (after culture for 6 h) by centrifugation. The cells were washed once with 200 ml of saline. About 7 g of packed cells was obtained from 1 liter of culture; the cells were stored frozen at -20°. E. coli NIH J was cultivated in nutrient broth.

Antibiotic Assay — The amount of polymyxin E (a mixture of polymyxin E_1 and E_2) produced by cultures of A. polyaerogenes was determined by bioassay using E. coli NIH J as a test organism. A 20-ml portion of the culture was withdrawn from the flask at various times, and the pH was adjusted to 2.0 by the addition of 4 N H_2SO_4. After removal of the cells by centrifugation at 20,000 x g for 15 min, the pH of the supernatant fluid containing polymyxin E was adjusted to 7.0 with 5 N NaOH. The fluid was filtered through a Millipore membrane filter (0.45 μm pore size). The sterilized fluid and a standard solution of polymyxin E sulfate (100 units, 25 μg/ml) were serially diluted in powers of 2 with sterilized distilled water. E. coli NIH J grown in nutrient broth at 37° for 6 h (about 420 Klett units at 660 nm) was diluted 10-fold with fresh broth. A mixture of 1.0 ml of the test solution or the standard polymyxin E solution, 4 ml of nutrient broth and 0.03 ml of the diluted cell suspension of E. coli was incubated at 37° for 18 h, and the minimum inhibitory concentration of the sample was determined.

Assay of Amino Acid Activating Enzymes — Amino acid activation was determined by measuring the exchange of $^{32}PP_i$ with ATP according to the method of Roth and Ames (16) with some modifications. The reaction mixture contained, in a final volume of 0.4 ml, 40 μmoles of Tris-HCl buffer (pH 8.0), 2 μmoles of $MgCl_2$, 1 μmole of ATP, 1 μmole of $[^{32}P]K_4P_2O_7$ (about 50,000 cpm), 1 μmole of dithiothreitol, 2 μmoles of a substrate amino acid and an appropriate amount of enzyme protein. The mixture was incubated at 30° for 30 min. At the end of incubation, 12 mg of charcoal (acid-washed Norit A) in 0.25 ml of 0.8 M $HClO_4$ was added to the reaction mixture. The mixture was poured onto a Millipore membrane filter (0.45 μm pore size). The filter was washed six times with 5 ml of 0.8 M $HClO_3$ and ten times with 10 ml of cold water. The filter was dried, placed in 5 ml of scintillation fluid and the radioactivity was determined in a Beckman liquid scintillation counter.

Isolation of an Enzyme-substrate Complex by Sephadex G-50 Column chromatography — The reaction mixture contained the following in a total volume of 0.2 ml. (A) 10 μmoles of Tris-HCl buffer (pH 8.0), 0.8 μmole of $MgCl_2$, 0.4 μmole of ATP, 1 μmole of dithiothreitol, 10 μg of inorganic pyrophosphatase, 28.9 nmoles of DL-[^{14}C]DAB (17.3 μC_i/μmole) and 216 μg of D-II fraction protein; (B) 10 μmoles of Tris-HCl buffer (pH 8.0), 0.8 μmole of $MgCl_2$, 24.5 nmoles of [2,8-^{3}H]ATP (199 μC_i/μmole), 1 μmole of dithiothreitol, 10 μg of inorganic pyrophosphatase, 1 μmole of L-DAB and 435 μg of D-II fraction protein. The mixture was incubated at 30° for 15 min, and then applied to a Sephadex G-50 column (1 x 25 cm) previously equilibrated with 0.05 M citrate buffer (pH 5.6) containing 4 mM $MgCl_2$ and 1 mM dithiothreitol. The column was eluted with the same buffer and 0.25 ml fractions were collected. An aliquot of 50 μl was applied to a Toyo No. 5C filter paper and the radioactivity

was determined in a Beckman liquid scintillation counter.

Assay for amino acid binding to the enzyme protein — The reaction mixture contained, in a final volume of 0.1 ml, 5 µmoles of Tris-Hcl buffer (pH 8.0), 0.4 µmole of $MgCl_2$, 0.2 µmole of ATP, 1 µmole of dithiothreitol, 5 µg of inorganic pyrophosphatase, a labelled amino acid(s) as indicated in the figure legends, and an appropriate amount of enzyme protein. The mixture was incubated at 30° for 15 min. The reaction was stopped by the addition of 2 ml of 7 % cold TCA. The reaction mixture was kept in an ice bath for 1 h, then poured onto a Millipore membrane filter. The filter was washed eight times with 5 ml of 0.02 M cold Tris-HCl buffer (pH 7.2). The filter was dried, placed in scintillation fluid, and the radioactivity was counted in a Beckman liquid scintillation counter.

Determination of protein concentration — Protein concentration was determined by the method of Lowry et al. (17) with crystalline bovine serum albumin as a standard.

RESULTS

Cell growth and polymyxin E production — Under the culture conditions described in "MATERIALS AND METHODS", the production of polymyxin E began at the late logarithmic phase of growth and increased rapidly to the maximum level within 3 h, amounting to 108 mg/liter (Fig. 1). L-DAB activating activity began to appear at

Fig. 1

the middle logarithmic phase of growth, prior to the production of polymyxin E, reached the maximum level and then decreased rapidly after the culture entered the stationary phase. These results were consistent with those of the studies on the production of polymyxin B (7, 12, 18) and other antibiotics (19-22).

Partial purification of L-DAB activating enzyme from Aerobacillus polyaerogenes — All operations were carried out at 0-4°.

Step 1. Preparation of crude extracts: The frozen cells (40 g) harvested at the late logarithmic phase of growth were suspended in 2 volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.02 M KCl, 1 mM dithiothreitol and 10 % sucrose (buffer A). After disruption of cells in 50-ml portions in a sonic disintegrator at 10 KHz for 10 min, the broken cell suspension was centrifuged at 18,000 x g for 30 min. The activity of the L-DAB activating enzyme in the crude extract prepared in the presence of 10 % sucrose was about 1.6 times that in the absence of sucrose. Sucrose protected L-DAB activating activity against heat inactivation and during storage of the enzyme.

Step 2. Centrifugation at 78,000 x g : The supernatant fluid at 18,000 x g was centrifuged at 78,000 x g for 90 min and the precipitate was discarded.

Step. 3. Ammonium sulfate fractionation : Solid ammonium sulfate was added (28 g per 100 ml of the supernatant solution) to the above supernatant solution, and the precipitate was collected by centrifugation at 20,000 x g for 20 min. The pellets, which contained 630 mg of protein, were dissolved in about 15 ml of buffer A. The enzyme solution was dialyzed overnight against 1 liter of buffer A.

Step 4. DEAE-cellulose column chromatography : The dialyzed ammonium sulfate fraction was applied to a DEAE-cellulose column (3 x 13 cm, Cl^- form) equilibrated with buffer A. The column was eluted as shown in Fig. 2. Nine milliliter fractions were collected.

Fig. 2

DAB activating activity was found in the 2nd and 3rd peaks in the elution profile of proteins. Fractions 27 through 35 were combined to give D-I fraction and fractions 67 through 75 to give D-II fraction. It should be noted that L-leucine-dependent ATP-PP_i exchange activity coincided with the peaks of L-DAB activating activity. Both fractions were brought to 0.45 saturation of ammonium sulfate and each precipitate was collected by centrifugation at 20,000 x g for 20 min, then dissolved in about 10 ml of buffer A containing 10 % glycerol. The enzyme fractions were dialyzed against the above buffer for 3 hours and could be stored at -20° without significant

loss of activitv for at least a month. The result of a typical purification is shown in Table I. The specific L-DAB activation

Table I

of D-II fraction was 23 times higher than that of the 78,000 x g supernatant solution. D-II fraction was used throughout the following experiments.

ATP-PP_i exchange activities dependent on constituent amino acids of polymyxin E — Table II shows that D-II fraction catalyzed the ATP-PP_i exchange reaction dependent on L-DAB, L-leucine and L-threonine, which are constituent amino acids of polymyxin E, but it did not catalyze the ATP-PP_i exchange reaction dependent on amino acids analogous to L-DAB, i. e., L-ornithine, L-lysine, γ-amino-n-butyric acid and L-α-amino-n-butyric acid. D-Leucine is a constituent of polymyxin E, but was not activated. The L-valine-dependnet ATP-PP_i exchange observed may be due to valyl-tRNA synthetase.

Table II

D-II Fraction was subjected to Bio-gel P-300 gel filtration and each fraction obtained was assayed for ATP-PP_i exchange activities dependent on L-DAB, L-leucine and L-threonine. As shown in Fig. 3, a

major protein peak was found in fractions 18 though 29 (corresponding to the void volume), and each peak of ATP-PP_i exchange activity dependent on the three amino acids coincided with the protein peak. These results suggest that D-II fraction is involved in the biosynthesis of polymyxin E. The specific activity of L-DAB activating activity was increased 1.4-fold by this gel filtration.

Fig. 3

Isolation of enzyme-bound DAB and adenyl-enzyme complex — When D-II fraction was incubated with DL-[^{14}C]DAB in the presence of ATP and Mg^{2+} ions, an enzyme-amino acid complex was formed and could be isolated by Sephadex G-50 column chromatography (Fig. 4A). It was

Fig. 4

shown that the adenine moiety of ATP was bound to the enzyme protein, when [^{3}H]ATP and L-DAB were used as substrates (Fig. 4B). The isolated enzyme-DAB and adenyl-enzyme complexes were precipitated with TCA as described in Table III. While about 40 % of [^{14}C]DAB in the Sephadex eluate remained in the TCA precipitated meterial, in the experiment with [^{3}H]ATP only the supernatant fraction contained a radioactive compound. When the enzyme was incubated with β,γ-[^{32}P]ATP in place of [^{3}H]ATP, no radioactive enzyme-substrate complex was formed

Table III

(data not shown). These results indicate that a half of the DAB bound to the enzyme was in an acid-stable form and the rest was acid-labile DAB adenylate.

The Nature of the Bond Between DAB and the Enzyme — When the TCA-precipitated enzyme-[^{14}C]DAB complex was incubated in an alkaline solution or 1 % mercuric acetate solution, most of the remaining radioactivity was released from the enzyme protein (Table IV). This

Table IV

result indicates that DAB was bound to the enzyme protein as a thioester.

Requirement for Enzyme-DAB Complex Formation — Enzyme-DAB complex formation was determined by a membrane filter method. The binding of DAB to the enzyme required ATP, Mg^{2+} ions and dithiothreitol (Table V).

Table V

Effect of Protein Concentration on the Binding of DAB to the Enzyme — A linear relationship was found between the amount of [^{14}C]DAB bound to D-II fraction and the amount of D-II fraction, as

shown in Fig. 5.

Fig. 5

Binding of L-leucine and L-threonine to the Enzyme — The binding of L-leucine and L-threonine to D-II fraction protein was also observed (Table VI). They were released from the enzyme protein by mild alkali treatment like DAB (data not shown). The molar ratio of DAB, leucine

Table VI

and threonine bound to D-II fraction was approximately 3 : 1 : 1.4. In the presence of two or three amino acids, the radioactivity bound to D-II fraction showed in an additive tendency, but did not exceed the sum of the radioactivities of the individual amino acids bound to the enzyme (Table VII). These results suggest that the three component amino acids of polymyxin E were bound to the corresponding active sites of the enzyme and that no intermediary peptides bound to the enzyme were formed during incubation.

Table VII

DISCUSSION

L-DAB is an unusual amino acid which is not found in proteins. It was shown that radioactive L-DAB was incorporated into polymyxin B by growing cultures of a polymyxin B producing organism, B. polymyxa strain Pfizer 2459 (18). A cell-free extract that catalyzed an L-DAB-dependent exchange of ATP and PP_i was prepared from the same organism (10-12). However, this enzyme activity was reported not to be detected in extracts of polymyxin D (23) and colistin (polymyxin E) (9) producing organisms.

We found an L-DAB activating enzyme in another polymyxin E producing organism, A. polyaerogenes and purified it partially. The purified enzyme activated also L-leucine and L-threonine, both of which are the constituent amino acids of polymyxin E. The enzyme bound all three amino acids as thioesters, like those synthetases of gramicidin S, tyrocidines, bacitracins and gramicidin A (24-27). These results, together with the earlier findings that polymyxin synthesis was not inhibited by inhibitors of protein synthesis (7-9), suggest that polymyxin is synthesized by the multienzyme thiotemplate mechanism (1-6).

In view of the presence of fatty acids (MOA or IOA) conjugated with the N-terminal DAB, the synthesis of an enzyme-bound acyl-DAB may be considered as a prerequisite for the initial peptide bond formation and subsequent elongation of the chain. In the case of gramicidin A synthesis, it was shown that the synthesis of an enzyme-bound formylvaline initiated the subsequent peptide bond formation (27). Ito et al. (13) reported that the addition of MOA-DAB or IOA-DAB together with DAB and L-leucine to a reaction mixture consisting of soluble extracts of B. colistinus and an ATP generating system enhanced three-fold the incorporation of L-[^{14}C]threonine into colistins. However, the role

of MOA-DAB or IOA-DAB is not clear, because a control experiment in which only MOA-DAB or IOA-DAB was omitted was not carried out. We tried to synthesize polymyxin E by incubating IOA-DAB together with DL-[^{14}C]DAB, L-leucine, L-threonine, ATP and crude extracts of A. polyaerogenes without success.

There are three possible ways of introducing an acyl moiety into peptides: the first is the synthesis of a free acyl amino acid followed by its activation and incorporation into peptides, the second is acylation of an intermediary or completed peptide and the third is activation and thioesterification of an amino acid followed by acylation and peptidation. The incorporation of acetylleucine and acetylleucyl= leucine into leupeptin acid was reported recently by Hori et al. (28). The above-described result by Ito et al. (13) may support such a possi= bility in polymyxin synthesis. Acetylation of various proteins may be considered to belong to the second mechanism, but there is no knowledge available on the mechanism of acylation of small preformed peptides. The third mechanism was recently revealed to be involved in the biosyn= thesis of gramicidin A. Akashi and Kurahashi (27, 29, 30) presented evidence that the initiation of gramicidin A synthesis occurs with formylation of valine thioesterified to a component of gramicidin A synthetase, followed by peptide elongation via the formation of formyl= valylglycine. The synthesis of N-methylvaline by methylation of valine thioesterified to enniatin B synthetase prior to the peptide bond for= mation (31) is analogous to this mechanism. There are some analogy between these reactions and formylmethionine synthesis for the initi= ation of protein synthesis that occurs while methionine is bound to $tRNA^{fMet}$.

At present we do not have any experimental evidence that rules out

the first two possibilities in the synthesis of the acyl-DAB moiety of polymyxins, but we are inclined to adopt the third mechanism as a working hypothesis upon which we experiment on the total synthesis of polymyxin E by a cell-free enzyme system.

We thank Dr. K. Hayashi of Kyoto University and Dr. Y. Kimura of Mukogawa Women's University for supplying strains of bacteria and Mr. H. Takeda of Banyu Pharmaceutical Co. Ltd. for a generous gift of polymyxin E sulfate.

REFERENCES

1. Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445-459
2. Kurahashi, K. in Antibiotics. Biosynthesis (Corcoran, J.W., ed.) Vol. IV, Springer-Verlag, Berlin·Heidelberg·New York. in press
3. Laland, S.G. & Zimmer, T.-L. (1973) in Essays in Biochemistry (Campbell, P.N. & Greville, G.D. eds.) Vol. 9, pp 31-57, Academic Press, London
4. Lipmann, F. (1971) Science 173, 875-884
5. Kleinkauf, H. & Koischwitz, H. (1978) Prog. Mol. Subcellular Biol. 6, 59-112 (1978)
6. Katz, E.& Demain, A.L. (1977) Bacteriol. Rev. 41, 449-474
7. Daniels, M.J. (1968) Biochim. Biophys. Acta 156, 119-127
8. Monreal, J. & Paulus, H. (1970) Biochim. Biophys. Acta 199, 280-282
9. Ito, M., Aida, K.,& Uemura, T. (1970) Biochim. Biophys. Acta 213, 244-247
10. Ciferri, O., DiGirolamo. M., & DiGirolamo, A.B. (1961) Nature 191, 411
11. Brenner, M., Gray, E., & Paulus, H. (1964) Biochim. Biophys. Acta 90, 401-403
12. Jayaraman, K., Monreal, J., & Paulus, H. (1969) Biochim. Biophys. Acta 185, 447-457
13. Ito, M., Koyama, Y., Aida, K., & Uemura. T. (1970) Biochim. Biophys. Acta 215, 418-420
14. Suzuki, T., Hayashi, K., Fujikawa, K., & Tsukamoto, K. (1965) J. Biochem. 57, 226-227
15. Nishikawa, K., Hosoi, K., Suzuki, J., Yoshimura, S., & Horio, T. (1973) J. Biochem. 73, 537-553
16. Roth, J.R. & Ames, B.M. (1966) J. Mol. Biol. 22, 325-334

17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

18. Paulus, H. & Gray, E. (1964) J. Biol. Chem. 239, 865-871

19. Tomino, S., Yamada, M., Itoh, H., & Kurahashi, K. (1967) Biochemistry 6, 2552-2560

20. Yamada, M. & Kurahashi, K. (1968) J. Biochem. 63, 59-69

21. Fujikawa, K., Suzuki, T., & Kurahashi, K. (1968) Biochim. Biophys. Acta 161, 232-246

22. Frøyshov, Ø. & Laland. S.G. (1974) Eur. J. Biochem. 46, 235-242

23. DiGirolamo, M., Ciferri, O., DiGirolamo, A.B., & Albertini, A. (1964) J. Biol. Chem. 239, 502-507

24. Gevers, W., Kleinkauf, H., & Lipmann, F. (1969) Proc. Natl. Acad. Sci. U.S. 63, 1335-1342

25. Roskoski, R., Jr., Gevers, W., Kleinkauf, H., & Lipmann, F. (1970) Biochemistry 9, 4839-4845

26. Ishihara, H. &. Shimura, K. (1974) Biochim. Biophys. Acta 338, 588-600

27. Akashi, K. & Kurahashi, K. (1978) J. Biochem. 83, 1219-1229

28. Hori, M., Hemmi, H., Suzukake, K., Hayashi, H., Uehara, Y., Takeuchi, T., & Umezawa, H. (1978) J. Antibiotics 31, 95-98

29. Akashi, K., Kubota, K., & Kurahashi, K. (1977) J. Biochem. 81, 269-272

30. Akashi, K. & Kurahashi, K. (1977) Biochem. Biophys. Res. Commun. 77, 259-267

31. Zocher, R. & Kleinkauf, H. (1978) Biochem. Biophys. Res. Commun. 81, 1162-1167

Table I. Purification of the L-DAB activating enzyme. Experimental procedures are described in "RESULTS".

Fraction	Protein (mg)	Specific activity[a]	Yield (%)
1. 78,000 x g supernatant solution	1,850	0.33	100
2. $(NH_4)_2SO_4$ fraction	636	1.08	112
3. D-I fraction	68	0.62	7
D-II fraction	12	7.50	14

[a] Units/mg of protein; one unit of activity is defined as that amount of enzyme which exchanges 1 μmole of PP_i with ATP in 30 min under the conditions described in "MATERIALS AND METHODS".

Table II. Amino acid-dependent ATP-PP_i exchange activities of D-II fraction. The ATP-PP_i exchange reaction was performed as described in "MATERIALS AND METHODS"; 46 μg of D-II fraction protein was used.

Amino acid	$^{32}PP_i$ incorporated into ATP (nmoles)
L-DAB	144.1
L-Leucine	86.7
L-Threonine	35.5
D-Leucine	4.2
L-Methionine	5.5
L-Valine	27.3
L-Ornithine	1.9
L-Lysine	1.0
L-α-Amino-n-butyric acid	2.7
γ-Amino-n-butyric acid	0.4

Table III. Acid treatment of the enzyme-substrate complex. About 2 ml of the eluate that contained the protein-bound [^{14}C]DAB or the protein-bound [^{3}H]adenyl moiety as shown in Fig. 4A and 4B, was treated with TCA at a final concentration of 7 %. The precipitate was washed once with 3 ml of 7 % TCA and the precipitant was extracted with 2 ml of ethanol-ether (3 : 1) and ether. The precipitated protein was dissolved in 200 µl of 0.01 N NaOH and an aliquot of 50 µl was applied to a Toyo No. 5C filter paper. The filter was dried and the radio= activity was determined in a Beckman scintillation counter using 5 ml of toluene-PPO scintillator solution.

Material	Radioactivity (cpm)	
	[^{14}C]DAB	[^{3}H]adenyl moiety
Sephadex eluate	1760 (100 %)	1865 (100 %)
TCA precipitate	724 (41 %)	84 (5 %)

Table IV. Liberation of [^{14}C]DAB from the enzyme-DAB complex. A sample of the enzyme-[^{14}C]DAB complex prepared and isolated as described in "MATERIALS AND METHODS" was divided into three equal portions. They were treated with TCA as described in the legend to Table III, and the precipitate was suspended in 0.1 ml of one of the following solutions: (1) 0.1 M glycine-NaOH buffer (pH 9.9), (2) 1 % mercuric acetate, and (3) distilled water. The suspension was incubated at 30° for 20 min and centrifuged at 2,000 x g for 5 min. 20 μl of the supernatant solution was applied to a Millipore filter. The filter was dried, placed in 5 ml of toluene-PPO scintillation fluid and the radioactivity was counted in a Beckman scintillation counter.

Treatment	Radioactivity released (%)
(1) Glycine-NaOH, pH 9.9	90
(2) Mercuric acetate, 1 %	72
(3) Distilled water	11

Table V. Requirement for enzyme-DAB complex formation. The experimental procedures are described in "MATERIALS AND METHODS". DL-[^{14}C]-DAB (17.3 μC_i/μmole, 28.9 nmoles) and 230 μg of D-II fraction protein were used.

Conditions	[^{14}C]DAB bound[a] (pmoles)
1. Complete	2.54
2. - Dithiothreitol	0.79
3. - Mg^{2+}	0.31
4. - ATP	0.48
5. - Enzyme	0.00

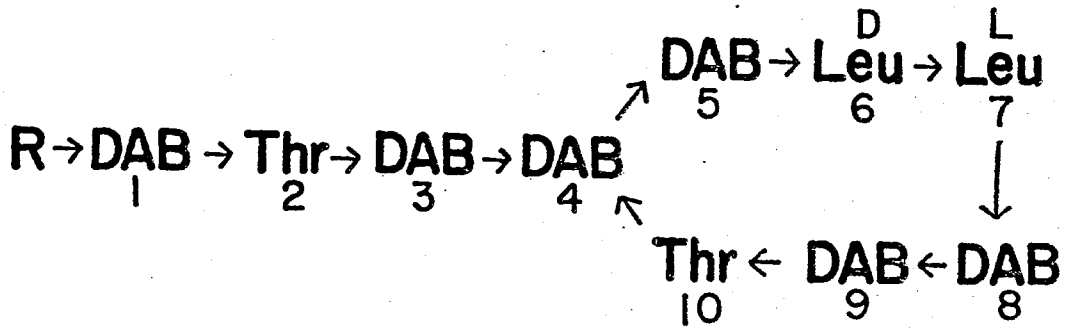
[a] The values of the control tubes (not incubated) were subtracted.

Table VI. Binding of DAB, L-leucine and L-threonine to the enzyme protein. The experimental procedures are described in "MATERIALS AND METHODS". L-[^{14}C]Leucine (50 $\mu C_i/\mu mole$, 5 nmoles), L-[^{14}C]threonine (50 $\mu C_i/\mu mole$, 5 nmoles), and DL-[^{14}C]DAB (17.3 $\mu C_i/\mu mole$, 14.5 nmoles) were used. In Experiment 1, 54 µg and in Experiment 2, 81 µg of D-II fraction protein was used.

Labelled amino acid used	Amino acid bound (pmoles)	
	Exp. 1	Exp. 2
DL-DAB	10.3	15.2
L-Leucine	3.2	5.1
L-Threonine	4.4	7.3

Table VII. Binding of amino acids to the enzyme protein in various combinations. The experimental conditions are described in "MATERIALS AND METHODS". L-[^{14}C]Leucine (50 $\mu C_i/\mu mole$, 5 nmoles), L-[^{14}C]threonine (50 $\mu C_i/\mu mole$, 5 nmoles), DL-[^{14}C]DAB (17.3 $\mu C_i/\mu mole$, 14.5 nmoles) and 81 µg of D-II fraction protein were used.

Labelled amino acid used	Amino acid bound (cpm)	(pmoles)
1. DL-DAB	878	16.0
2. L-Leucine	697	5.5
3. L-Threonine	1061	9.2
4. DL-DAB + L-leucine	1488	
5. DL-DAB + L-threonine	1548	
6. DL-DAB + L-leucine + L-threonine	2170	
7. L-Leucine + L-threonine	1436	



Polymyxin E_1 : R=6-methyloctanoic acid (MOA)
Polymyxin E_2 : R=isooctanoic acid (IOA)
Polymyxin E_3 : R=octanoic acid (OA)
DAB : 2,4-diaminobutyric acid

Diagram 1. Structure of Polymyxin E

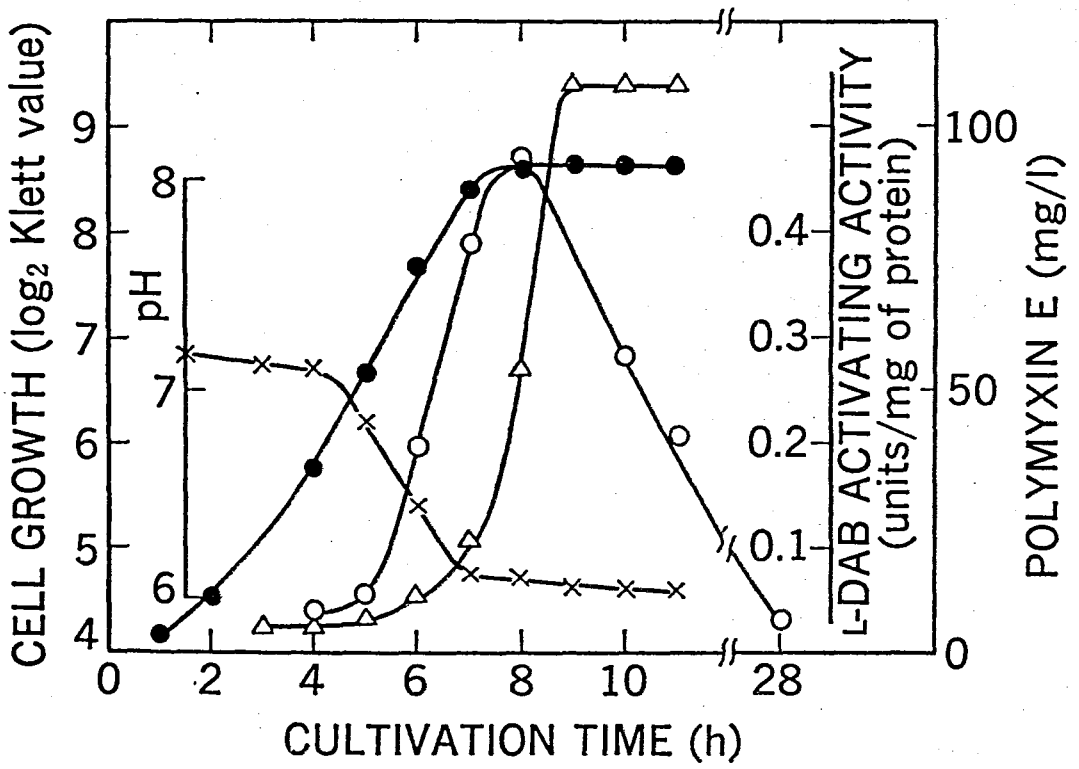


Fig. 1. Polymyxin E production and L-DAB activating activity in the relation to the growth of A. polyaerogenes cells. The turbidity of the cells grown as described in "MATERIALS AND METHODS" was measured with a Klett-summerson colorimeter. Polymyxin E in the culture and ATP-PP_i exchange activity dependent on L-DAB were determined as described in "MATERIALS AND METHODS". -●-, Cell growth; -o-, L-DAB activating activity; -Δ-, polymyxin E production; -x-, pH of the medium.

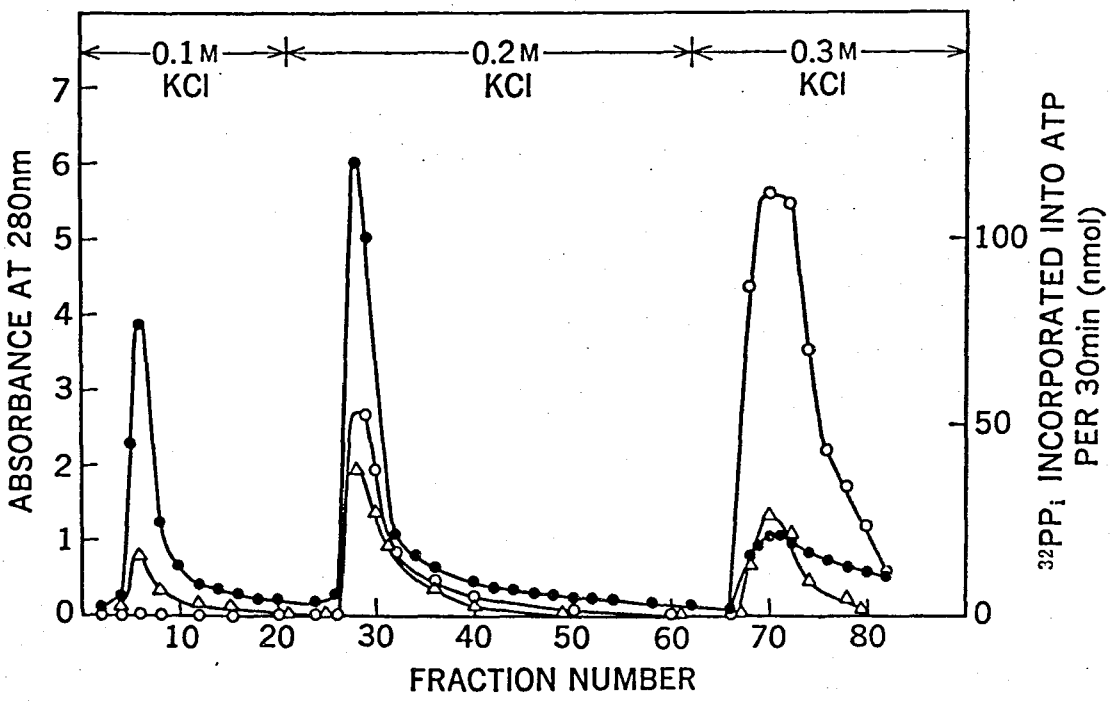


Fig. 2. DEAE-cellulose column chromatography of the L-DAB activating enzyme. The elution of the enzyme protein was carried out as described in "RESULTS". A sample of 100 µl of each fraction was assayed for ATP-PP_i exchange activity dependent on L-DAB and L-leucine. -●-, Absorbance at 280 nm; -o-, L-DAB-dependent exchange activity; -△-, L-leucine-dependent exchange activity.

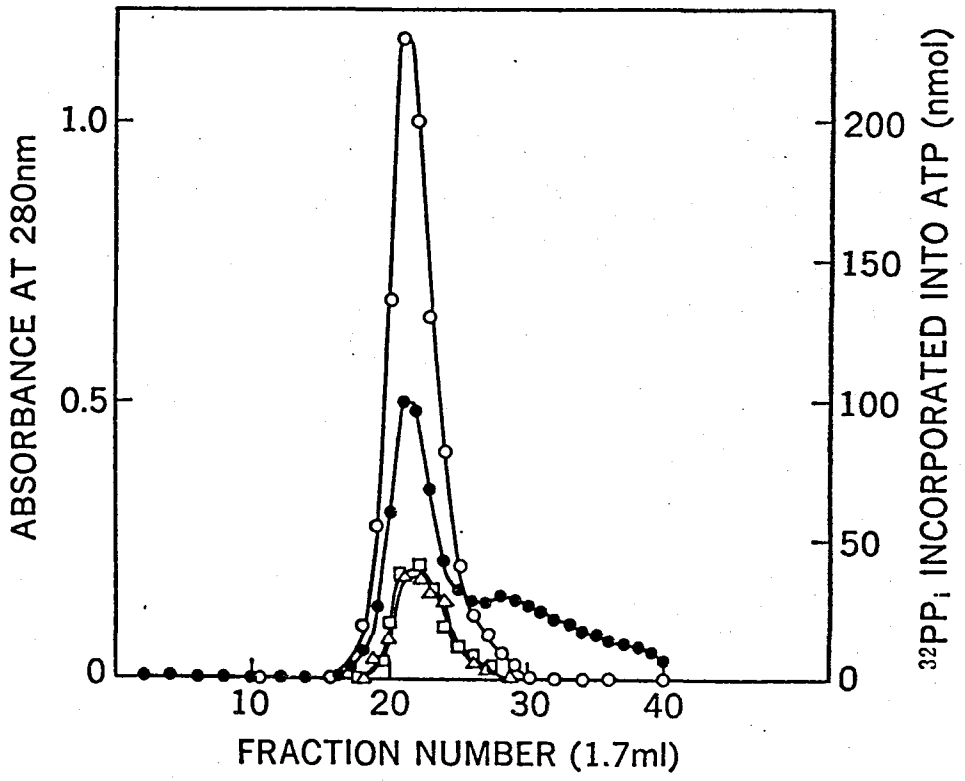


Fig. 3. ATP-PP_i exchange activities dependent on L-DAB, L-leucine and L-threonine in Bio-gel P-300 gel filtration fractions. D-II fraction (4.4 mg of protein, 1.2 ml) was applied to a Bio-gel P-300 column (1 x 100 cm, 133 ml) which had been equilibrated with buffer A, and 1.7 ml fractions were collected. 30 µl of each fraction was assayed for ATP-PP_i exchange activities dependent on the amino acid indicated. -●-, Absorbance at 280 nm; -o-, L-DAB-dependent exchange activity; -Δ-, L-leucine-dependent exchange activity; -□-, L-threonine-dependent exchange activity.

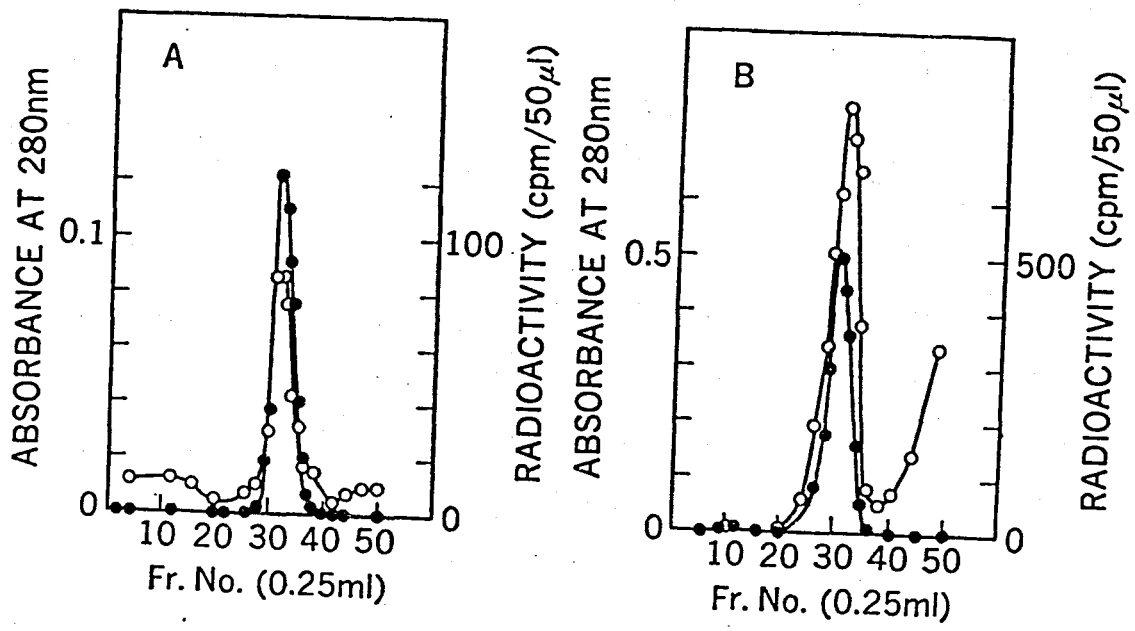


Fig. 4. Isolation of the enzyme-bound substrate. The experimental procedures are described in "MATERIALS AND METHODS". A. DL-[^{14}C]DAB and unlabeled ATP as substrates. -●-, Absorbance at 280 nm; -o-, radioactivity of [^{14}C]DAB. B. Unlabeled DAB and [^{3}H]ATP as substrates. -●-, Absorbance at 280 nm; -o-, radioactivity of [^{3}H]ATP.

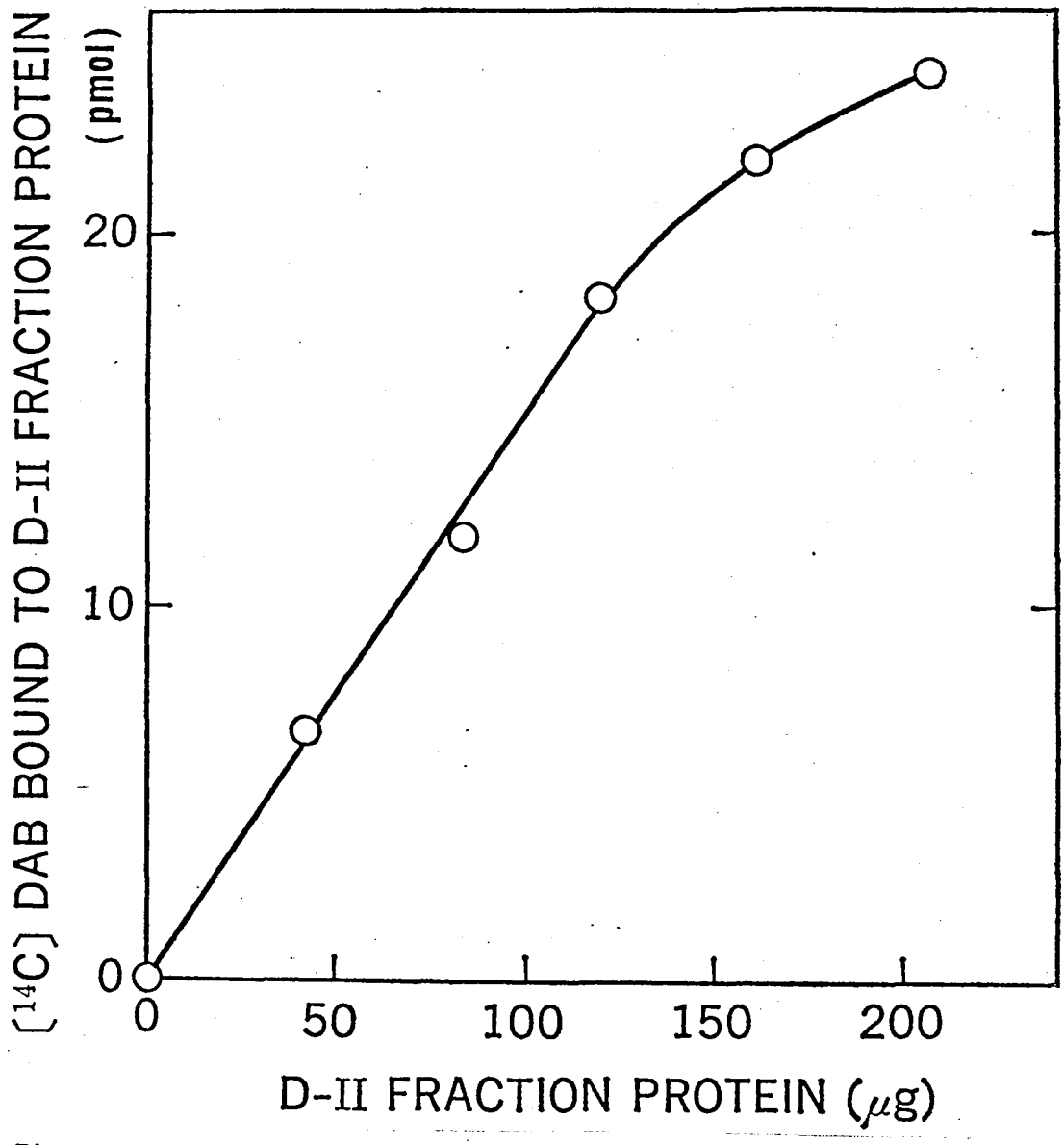


Fig. 5. Effect of protein concentration on the binding of [^{14}C]DAB to D-II fraction protein. The experimental procedures are described in "MATERIALS AND METHODS".

PART II

Synthesis of Enzyme-bound N^2-Octanoyl-L-2,4-diaminobutyric Acid

(A) Preliminary evidence for enzymatic synthesis of enzyme-bound octanoyldiaminobutyric acid

A partially purified enzyme of Aerobacillus polyaerogenes which elaborates polymyxin E activates L-2,4-diaminobutyric acid and binds it as a thioester. The incubation of the enzyme preparation with octanoyl coenzyme A and L-2,4-diaminobutyric acid in the presence of ATP and an ammonium sulfate fraction yields octanoyldiaminobutyric acid thioesterified to the enzyme.

Polymyxin E (colistin) is a peptide antibiotic produced by Bacillus colistinus or Aerobacillus polyaerogenes (1, 2). Its structure is shown in Diagram 1. The amino terminal L-2,4-diaminobutyric acid

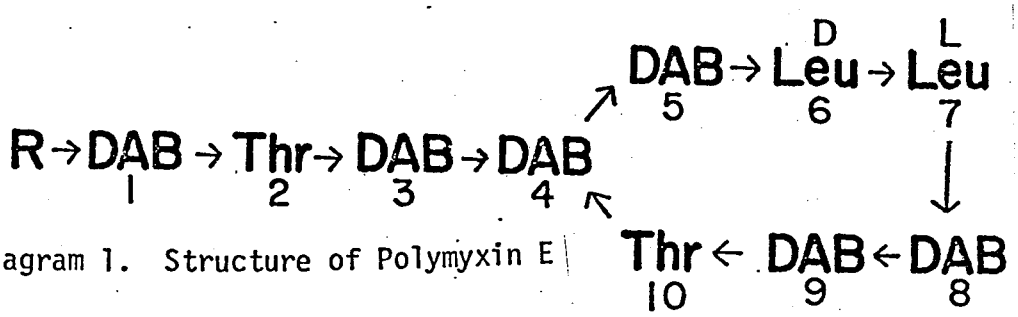


Diagram 1. Structure of Polymyxin E

(DAB) is acylated with either of 6-methyloctanoic acid (MOA), isooctanoic acid (IOA) or octanoic acid (OA) (3).

Ito et al. (4) reported that an incubation of N^2-6-methyloctanoyl (or isooctanoyl)-2,4-diaminobutyric acid together with the component amino acids, ATP and crude extracts of B. colistinus Koyama gave rise to a compound which was identified as colistin (polymyxin E) by paper chromatography. However, the details of the mechanism of biosynthesis of polymyxin series of antibiotics have remained unclarified. The presence of a DAB activating enzyme in the polymyxin B producing organism, Bacillus polymyxa 2459 and the polymyxin E producing organism A. polyaerogenes was reported by Jayaraman et al. (5) and Komura and Kurahashi (1). The partially

purified enzyme of A. polyaerogenes was shown to activate in addition L-leucine and L-threonine and bind all three amino acids as thioesters (1). In this communication evidence that DAB thioesterified to the DAB activating enzyme is acylated with octanoyl CoA is presented.

Preparation of DAB Activating Enzyme ——— Crude extracts and an ammonium sulfate fraction (AS-fraction) of A. polyaerogenes were prepared as described previously (1) with an exception that 0.1 M Tris-HCl buffer (pH 7.4) containing 0.01 M KCl, 5 mM dithiothreitol (DTT) and 10 % sucrose (buffer A') was used in the place of buffer A. The DAB activating enzyme was purified further by Sepharose 4B gel-filtration as follows. AS-fraction (17.7 ml, 499 mg of protein) was applied to a Sepharose 4B column (4 x 70 cm, 879 ml) previously equilibrated with buffer A'. The DAB activating enzyme was eluted with buffer A' at a flow rate of 24 ml/h, and 8 ml fractions were collected. Each effluent fraction was immediately mixed with glycerol at a final concentration of 10 % to prevent the loss of enzyme activity. The fractions (eluted at Ve/Vo=1.6) which contained ATP-$^{32}PP_i$ exchange activities dependent on L-DAB, L-leucine and L-threonine were pooled. The pooled fraction (153 ml) was brought to 0.5 saturation of ammonium sulfate and the precipitated protein was dissolved in buffer A' containing 10 % glycerol. Two batches of Sepharose 4B gel-filtration fraction were combined and the combined fraction (8.4 ml, 119.7 mg of protein) was gel-filtrated again through the same column of Sepharose 4B. Fig. 1

Fig. 1

shows the ATP-$^{32}PP_i$ exchange activities dependent on the component amino acids of polymyxin E. The peaks of L-DAB, L-leucine and L-threonine activating activities coincided with each other. Fractions 62 through 74 were pooled and protein was precipitated by the addition of ammonium sulfate to 0.5 saturation. The precipitate was dissolved in 2 ml of buffer A, containing 50 % glycerol. The specific L-DAB activating activity of the second Sepharose 4B fraction was 5.3 units per mg of protein and 29 times higher than that of the crude extracts. One unit of activity is defined as that amount of enzyme which exchanges 1 μmol of PP_i with ATP in 30 min under the conditions described in a previous paper (1). The enzyme solution was stored at -70°C without a significant loss of activity for at least a month and used in the following experiments. In some cases, AS-fraction was only once gel-filtrated through a large size column of Sepharose 4B with a bed volume of 100-fold larger than the volumn of AS-fraction applied. This Sepharose 4B gel-filtration fraction had a similar specific DAB activating activity to that of the above preparation and L-luecine and L-threonine activating activities which were involved in the biosynthesis of polymyxin E could be separated from the leucyl-tRNA synthetase or threonyl-tRNA synthetase. The approximate molecular weight of the enzyme was estimated to be 300,000 by means of sucrose density gradient centrifugation (data will be presented elsewhere).

Binding of DAB, L-leucine and L-Threonine by the Enzyme ——— As shown in Table I, the partially purified enzyme bound the three component amino acids of polymyxin E in a molar ratio of 6 : 2 : 3.

Table I

Synthesis and Isolation of Enzyme-bound N^2-Octanoyl-2,4-diaminobutyric acid (OA-DAB) ——— As described in the legend to Fig. 2, octanoyl CoA was incubated together with [4-^{14}C]DAB and other ingredients necessary for the binding reaction of DAB to the enzyme. A small amount of the ammonium sulfate fraction was added as a source of transacylase. Fig. 2 shows the radiochromatogram of the products

Fig. 2

released from the trichloracetic acid precipitated enzyme protein by a mild alkali treatment. A radioactive peak (Rf=0.79) corresponding to the area of an authentic sample of OA-DAB was observed. There was also a small peak of radioactivity corresponding to L-3-amino=pyrrolid-2-one (pyrrolidone), but no [^{14}C]DAB was recovered. We consider that pyrrolidone and the large radioactive peak followed the peak of OA-DAB were derived from [^{14}C]DAB thioesterified to the enzyme upon alkali hydrolysis. The latter compound passed through a Dowex 1 (OH^--form) column and upon acid hydrolysis only [^{14}C]DAB was recovered, suggesting that it may be the diketopiperazine of DAB.

Release of DAB from the Product by Acid Hydrolysis ——— The

area indicated by I in Fig. 2 was cut and eluted. The eluate was condensed and rechromatographed as shown in Fig. 3. A single radioactive peak (Rf=0.81) corresponding to the OA-DAB marker was observed. In Fig. 4, the radiochromatogram of the hydrolyzate

Fig. 3

Fig. 4

of the product rechromatographed and eluted is shown. [^{14}C]DAB was recovered from the product.

It was also observed that [1-^{14}C]OA was incorporated into the product which comigrated with an authentic sample of OA-DAB, when [1-^{14}C]OA-CoA was used as an acyl donor (data not shown).

It was reported previously from this laboratory that formylation of valine thioesterified to the valine activating enzyme is prerequisite to peptide chain elongation in the biosynthesis of gramicidin A (6, 7). The results presented in this communication suggest that the initiation of polymyxin biosynthesis also takes place with the initial formation of acyl-DAB bound to the DAB activating enzyme. Incubation of DAB together with [^{14}C]threonine under the conditions of the binding experiments did not give rise to any enzyme-bound intermediary peptides (unpublished data).

Recently Mohr and Kleinkauf (8) reported that enzyme-bound acetylamino=isobutyric acid was formed by alamethicin synthetase. It was shown by Zocher and Kleinkauf (9) that methylation of enzyme-bound valine took place during the synthesis of enniatin B. Modification of amino acids at the enzyme-bound stage with acyl, methyl or some other moieties prior to the peptide bond formation may be a common feature of antibiotic peptide synthesis according to the multienzyme thio=template mechanism (10). Identification of octanoyl transferase and attempts to synthesize intermediary peptides of polymyxin E are under way.

We are very grateful to Dr. S. Sakakibara of the Peptide Institute, Protein Research Foundation and Dr. S. Wilkinson of the Wellcome Research Laboratories for supplying us N^2-octanoyl-2,4-diaminobutyric acid and L-3-aminopyrrolid-2-one, respectively, used in this work. We thank Mr. H. Takeda of Banyu Pharmaceutical Co. Ltd. for a generous gift of polymyxin E sulfate and Dr. K. Hayashi of Kyoto University and Dr. Y. Kimura of Mukogawa Women's University for supplying strains of bacteria.

REFERENCES

1. Komura, S. & Kurahashi, K. (1979) J. Biochem. 86, 1013-1021
2. Suzuki, T., Hayashi, K., Fujikawa, K., & Tsukamoto, K. (1965) J. Biochem. 57, 226-227
3. Withander, L. & Heding, H. (1976) J. Antibiotics 29, 774-775
4. Ito, M., Koyama, Y., Aida, K., & Uemura, T. (1970) Biochim. Biophys. Acta 215, 418-420
5. Jayaraman, K., Monreal, J., & Paulus, H. (1969) Biochim. Biophys. Acta 185, 447-457
6. Akashi, K., Kubota, K., & Kurahashi, K. (1977) J. Biochem. 81, 269-272
7. Akashi, K. & Kurahashi, K. (1978) J. Biochem. 83, 1219-1229
8. Mohr, H. & Kleinkauf, H. (1978) Biochim. Biophys. Acta 526, 375-386
9. Zocher, R. & Kleinkauf, H. (1978) Biochem. Biophys. Res. Commun. 81, 1162-1167
10. Kurahashi, K. in Antibiotics. Biosynthesis (Corcoran, J. W., ed.) Vol. IV, Springer-Verlag, Berlin-Heidelberg-New York, in press

Table I. Binding of DAB, L-leucine and L-threonine to the enzyme protein. The binding of [^{14}C]-amino acids to the enzyme protein was determined by a filter method described in a previous paper (1). 120 µg of the second Sepharose 4B gel-filtration fraction protein was used.

Labelled amino acids used	Amino acid bound (pmol)
DL-DAB	27.0
L-Leucine	9.0
L-Threonine	13.8

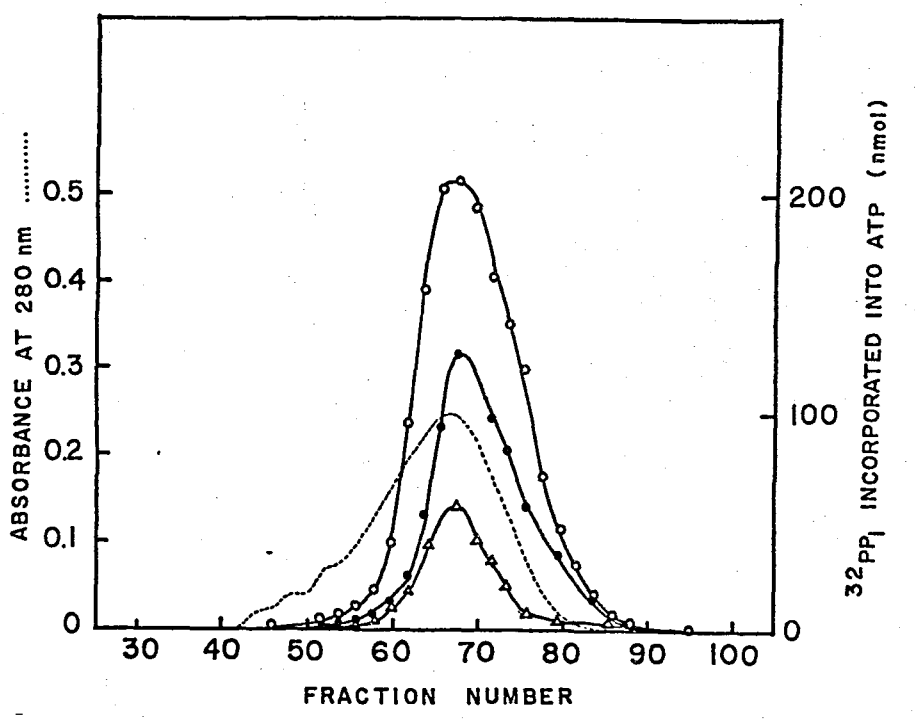


Fig. 1. ATP-PP_i exchange activities dependent on L-DAB, L-leucine and L-threonine of the second Sepharose 4B gel-filtration fractions. The first Sepharose 4B gel-filtration fraction (119.7 mg of protein, 8.4 ml) was applied to a Sepharose 4B column (4 x 70 cm, 879 ml) which had been equilibrated with buffer A' and 8 ml fractions were collected. 50 µl of each fraction was assayed for ATP-PP_i exchange activity dependent on the amino acids indicated. ----, Absorbance at 280 nm; -o-, L-DAB-dependent exchange activity; -●-, L-leucine-dependent exchange activity; -Δ-, L-threonine-dependent exchange activity.

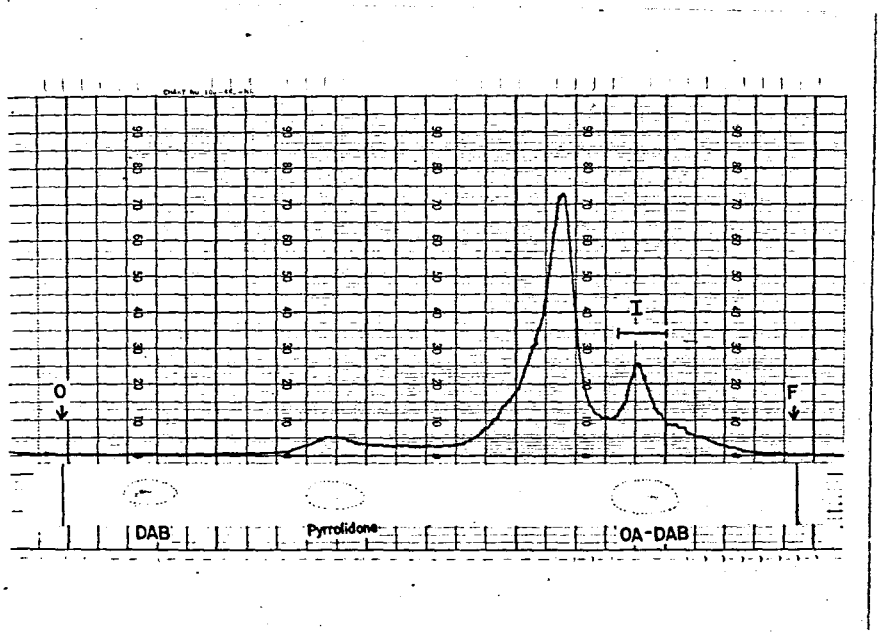


Fig. 2. Paperchromatography of radioactive products thioesterified to the enzyme. The reaction mixture contained the following components in a total volume of 0.18 ml: 10 µmol of Tris-HCl buffer (pH 7.7), 0.8 µmol of ATP, 2 µmol of $MgCl_2$, 1 µmol of DTT, 0.8 µmol of phosphoenolpyruvate, 0.4 µg of pyruvate kinase, 1 µg of inorganic pyrophosphatase 200 µg of ammonium sulfate fraction, 732 µg of enzyme protein and 90.1 nmol of DL-[4-^{14}C]-2,4-diaminobutyric acid (44 mC_i/mmol). The mixture was preincubated at 30°C for 5 min, and then octanoyl coenzyme A (0.2 µmol in 20 µl) was added. Incubation was continued at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of cold 7 % trichloroacetic acid (TCA) and 500 µg of bovine serum albumin as a carrier. The reaction mixture was kept in an ice bath for 1 h and the precipitated protein was collected by centrifugation. The precipitate was washed with 1 ml of cold 5 % TCA containing 0.5 mM L-DAB four times and the precipitant was extracted with 2 ml of an ethanol-ether mixture

(2 : 1) and of ether. The precipitated protein was dissolved in 200 µl of 0.01 N KOH and incubated at 30°C for 20 min. At the end of incubation, the tube was chilled in an ice bath and the pH was adjusted to 5.0 by the addition of 0.1 M perchloric acid. After centrifugation, the supernatant solution obtained was lyophilized. The dry powder was extracted with 100 µl of ethanol (99.5 %) twice, and ethanol was evaporated under a gentle stream of N_2 gas. The residue was dissolved in 50 µl of 50 % ethanol containing 10 µg of OA-DAB. Twenty identical incubations were carried out and combined for analysis by paperchromatography. Paperchromatography was carried out on Toyo No. 50 filter paper (2 x 40 cm) with a solvent system of n-butanol : acetic acid : H_2O (3 : 1 : 1). The radioactivity was scanned by a Packard radiochromatogram scanner Model 7201. The OA-DAB marker was visualized by ninhydrin spray.

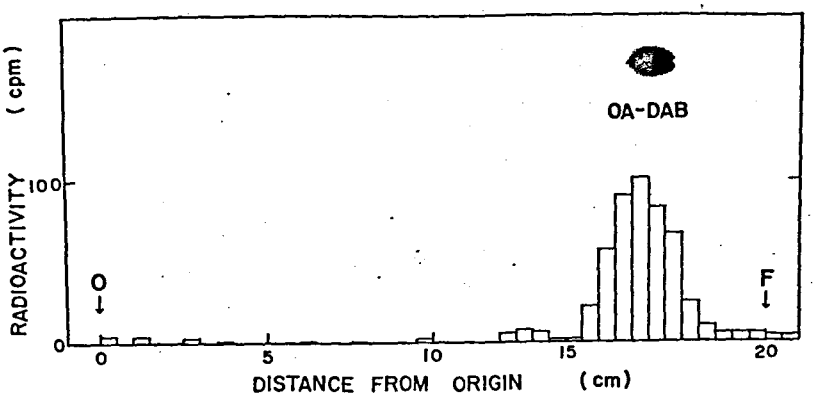


Fig. 3. The second paperchromatography of the product comigrating with OA-DAB. The radioactive area indicated by I in Fig. 2 was cut and eluted with 50 % ethanol (the total radioactivity of 17,000 cpm). A fortieth part of the eluate was chromatographed on Toyo No. 51A filter paper with a solvent system of n-butanol : acetic acid : pyridine : H_2O (15 : 3 : 10 : 12). The paper strip was cut lengthwise at every 5 mm and the radioactivity was counted in a Beckman liquid scintillation counter.

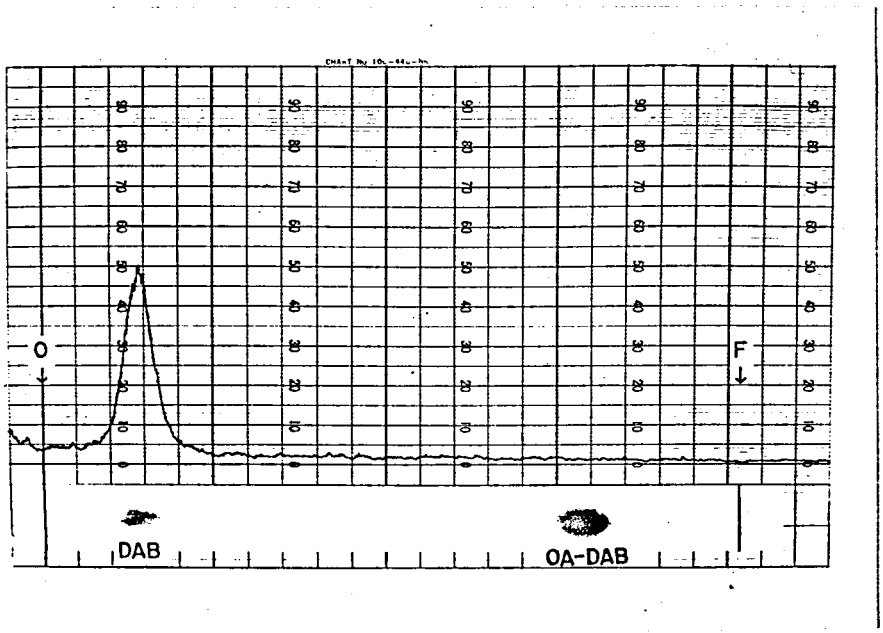


Fig. 4. Paperchromatography of acid hydrolyzate of the OA-DAB fraction. The remainder of the eluate described in Fig. 3 was chromatographed on Toyo No. 50 filter paper using the same solvent system as in Fig. 3. The single radioactive peak corresponding to OA-DAB was eluted with 50 % ethanol. The eluate was dried under vacuum. After hydrolysis in 100 µl of 3 N HCl at 100°C for 12 h, the hydrolyzate was chromatographed on Toyo No. 50 filter paper with the same solvent system as described in the legend to Fig. 2. The radioactivity was scanned by the Packard radiochromatogram scanner.

(B) Acylation of enzyme-bound L-2,4-diaminobutyric acid with octanoyl coenzyme A and its role in polymyxin E biosynthesis

---

Abbreviations : DAB, 2,4-diaminobutyric acid; OA, octanoic acid; MOA, 6-methyloctanoic acid; IOA, isooctanoic acid; OA-DAB, N^2-octanoyl-L-2,4-diaminobutyric acid; MOA-DAB, N^2-6-methyloctanoyl-L-2,4-diaminobutyric acid; IOA-DAB; N^2-isooctanoyl-L-2,4-diaminobutyric acid; TCA, trichloroacetic acid; OA-CoA, octanoyl coenzyme A.

A partially purified enzyme fraction, which catalyzed the ATP-PP_i exchange reaction dependent on three constituent amino acids of polymyxin E, was obtained from crude extracts of Aerobacillus polyaerogenes. The sedimentation coefficient of the enzyme was found to be 13.0 S by sucrose density gradient centrifugation. The incubation of the enzyme with octanoyl coenzyme A and diaminobutyric acid in the presence of ATP and an ammonium sulfate fraction brought about octanoyldiaminobutyric acid thioesterified to the enzyme protein. After mild alkali treatment, the enzyme-bound octanoyldiaminobutyric acid was released from the enzyme protein. The octanoyldiaminobutyric acid was identified by paper chromatography. After acid hydrolysis, diaminobutyric acid and octanoic acid were recovered at a molar ratio of 1 to 0.7. Acylating reaction of the enzyme-bound diaminobutyric acid requires an ammonium sulfate fraction as acyltransferase. When [^{14}C]threonine was incubated with L-2,4-diaminobutyric acid in the absence of octanoyl coenzyme A, no intermediary peptide was formed. These results suggest that acyldiaminobutyric acid bound to the enzyme protein is a possible initiation complex in the biosynthesis of polymyxin E.

A number of studies on the biosynthesis of peptide antibiotics such as gramicidin S, tyrocidines, bacitracins and gramicidin A revealed the multienzyme thiotemplate mechanism different from that of protein synthesis (1-6). Polymyxin E (colistin) is a peptide antibiotic produced by Bacillus colistinus or Aerobacillus polyaerogenes (7-9). Its structure is shown in diagram 1. The amino terminal L-2,4-diamino=

Diagram 1

butyric acid (DAB) is acylated with either 6-methyloctanoic acid (MOA), isooctanoic acid (IOA) or octanoic acid (OA) (10).

In order to clarify the biosynthetic mechanism of polymyxins by a cell-free enzyme system, an enzyme which catalyzed ATP-PP_i exchange reaction dependent on DAB was sought. Ciferri et al. (11), Brenner et al. (12), and Jayaraman et al. (13) previously described the presence of a DAB activating enzyme in cell-free extracts of polymyxin B producing bacteria (Bacillus polymyxa 2459). Komura and Kurahashi (9) also found such enzyme activity in cell-free extracts of polymyxin E producing bacteria. The partially purified enzyme of A. polyaerogenes was shown to activate in addition L-leucine and L-threonine and bind all three amino acids as thioesters, suggesting that the multienzyme thiotemplate mechanism may be operative in the biosynthesis of polymyxin E (9).

In our recent paper, it was shown that an enzyme-bound DAB was acylated in the presence of an ammonium sulfate fraction of crude extracts (AS-fraction) by octanoyl coenzyme A (14). This paper

describes the details of the transacylation reaction and discusses its role in the initiation of polymyxin biosynthesis.

MATERIALS AND METHODS

Chemicals and Enzymes — The following products were obtained commercially : L-2,4-diaminobutyric acid, octanoyl coenzyme A, coenzyme A, phosphoenolpyruvate, ATP and catalase [EC 1.11.1.6] from Sigma Chemical Co., DL-[1-^{14}C]2,4-diaminobutyric acid (44.0 mC_i/mmole) from CEA-France, [1-^{14}C]sodiumoctanoate (27.2 mC_i/mmole) from New England Nuclear, and inorganic pyrophosphatase [EC 3.6.1.1], pyruvate kinase [EC 2.7.1.40] and β-D-galactosidase[EC 3.2.1.23] from Boehringer Mannheim. [1-^{14}C]Octanoyl coenzyme A was enzymatically prepared according to the methods of Bar-Tana et al. (15) and purified by paper chromatography with a solvent system of ethanol-1 M ammonium acetate (5 : 2). N^2-Octanoyl-2,4-diaminobutyric acid, L-3-aminopyrrolid-2-one were the gifts from Dr. S. Sakakibara and Dr. S. Wilkinson, respectively.

Bacterial Strains and Growth — Polymyxin E producing A. polyaerogenes, which had been isolated by Dr. Hayashi from soils in Ikeda City, Osaka, was used in this study. The cells were cultured in a soluble starch medium and harvested at the late logarithmic phase of growth as described in a previous paper (9).

Enzyme Preparation — L-DAB activating enzyme was partially purified from sonicates of the cells by ultracentrifugation, ammonium sulfate fractionation and Sepharose 4B gel-filtration as described in earlier papers (9, 12). In this study, AS-fraction (14.6 ml, 735 mg of protein) was only once gel-filtrated through a Sepharose 4B column (5 x 78 cm, 1530 ml) previously equilibrated with buffer A'. The column was eluted with buffer A' at a flow rate of 24 ml/h, and 13 ml fractions were collected. Each effluent fraction was immediately mixed with glycerol to a final concentration of 10%. The active fractions were collected and concentrated as described in a

previous paper (12)

Enzyme Assays — (1) Assay of amino acid activating enzyme : Amino acid activation was determined by measuring the exchange of $^{32}PP_i$ with ATP according to the methods described in an earlier paper (9). (2) Assay of β-D-galactosidase: The enzyme activity was determined by the method of Pardee et al. (16). (3) Assay of catalase : The enzyme activity was spectrophotometricaly determined according to the method described by Chance (17).

Sucrose Density Gradient Centrifugation — Two tenths mililiter of the DAB activating enzyme mixed with 12.5 µg of catalase and 25 µg of β-D-galactosidase was layered on 11.6 ml of a 5-20 % sucrose density gradient in 50 mM potassium phosphate buffer, pH 7.1, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol, and centrifuged for 13.5 h at 32,000 rpm in a SW-41 rotor with a Beckman ultracentrifuge Model L5-65. Three tenths milliliter fractions were collected with an ISCO Model 640 density gradient fractionator.

Assay of OA-DAB Formation — The reaction mixture contained the following component in a total volume of 0.18 ml : 10 µmol of Tris-HCl buffer (pH 7.7), 0.8 µmol of phosphoenolpyruvate, 0.4 µmol of dithioth= reitol, 0.8 µmol of ATP, 2 µmol of $MgCl_2$, 0.4 µg of pyruvate kinase, 1 µg of inorganic pyrophosphatase, 9.01 nmol of DL-[4-^{14}C]DAB, an appropriate amount of Sepharose 4B fraction and AS-fraction as indicated in the figure legend. The mixture was preincubated at 30°C for 5 min, and then octanoyl coenzyme A (0.16 mol in 20 µl) was added. Incubation was continued at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of cold 7 % trichloroacetic acid (TCA) and 500 µg of bovine serum albumin was added as a carrier. The reaction mixture was kept in an ice bath for 1 h and the precipitated protein was collected by centrifugation. The precipitate was washed with 1 ml of cold 5 %

TCA containing 0.5 mM L-DAB twice and 1 ml of cold 2 % TCA twice. The precipitant was extracted with 2 ml of an ethanol-ether mixture (2 : 1) and of ether. The precipitated protein was dissolved in 0.2 ml of 0.01 N KOH and incubated at 30°C for 20 min. At the end of incubation, the tube was chilled in an ice bath and the pH was adjusted to 5 by the addition of 0.1 M perchloric acid. After centrifugation, the supernatant solution obtained was lyophilized. The dry powder was extracted with 0.1 ml of ethanol (99.5 %) twice, and ethanol was evaporated under a gentle stream of N_2 gas. The residue was dissolved in 50 µl of 50 % ethanol with 20 µg of OA-DAB as a carrier. The ethanol solution was applied to a Toyo No. 50 filter paper (2 X 40 cm) and chromatographed with Solvent B. The paper strip was cut lengthwise every 5 mm and the radioactivity in the area corresponding to OA-DAB was determined with a Beckman liquid scintillation counter.

Acid Hydrolysis of the Produsts labelled with [^{14}C]OA or both [^{14}C] OA and [^{14}C]DAB — A single product isolated by paper chromatography was eluted with 50 % ethanol and the eluate was evaporated to dryness. The residue was dissolved in 0.2 ml of 3 N HCl and 143 nmol of OA-DAB and 400 nmol of potassium octanoate were added as carriers. The tubes were evacuated, sealed and heated at 100°C for 12 h. After acid hydrolysis, the tubes were chilled at 0°C, and then the hydrolyzates were extracted three times with 0.4 ml of ethylether. (A) Ethereal phase: The pH of the ethereal fraction was adjusted to 8 by the addition of 10 mM KOH, and ether was removed under a gentle stream of N_2 gas. The residue was lyophilized and dry powder was extracted with 50 µl of ethanol (99.5 %) twice and subjected to paper chromatography for the analysis of OA. (B) Aqueous phase: The aqueous phase was dried under vacuum to remove HCl. The dry residue was dissolved in 50 µl of distilled water and

subjected to paper chromatography for the analysis of DAB.

Paper Chromatography — For the identification and quantitative determination of the acylated product, descending paper chromatgraphy was carried out with the following solvent systems.

Solvent A: n-butanol : acetic acid : H_2O = 3 : 1 : 1

Solvent B: n-butanol : acetic acid : pyridine : H_2O = 15 : 3 : 10 : 12

Paper chromatography of fatty acid was carried out ascendingly with the following solvent system.

Solvent C: ethanol : NH_4OH : H_2O = 80 : 5 : 15

Determination of Protein Concentration — Protein concentration was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.

RESULTS

Purification of DAB Activating Enzyme from A. polyaerogenes — DAB activating enzyme was purified as described in "MATERIALS AND METHODS". As shown in Fig. 1, the active fractions 57 through 71 possessed ATP-PP_i exchange activities dependent on all three constituent amino acids of polymyxin E, and each peak of ATP-PP_i exchange activity coincided with each other. This active fraction involved in polymyxin E biosynthesis was separated from leucyl-tRNA synthetase or threonyl-tRNA synthetase eluted in fractions 75 through 90. Purification and the recovery of the enzyme activity at each step are summarized in Table 1. Sepharose 4B fraction represented 23-fold purification over the crude extract, and the recovery of the activity was 37 %.

Fig. 1

Table 1

The sedimentation coefficient of the enzyme was determined by sucrose density gradient centrifugation as shown in Fig. 2. The S value was calculated to be 13.0 S, using catalase and β-galactosidase as standards. The apparent molecular weight of the enzyme was estimated to be about 300,000 using a value of molecular weight of 250,000 for catalase.

Fig. 2

Incorporation of [1-^{14}C]OA into OA-DAB — As described in "MATERIALS AND METHODS", enzymatically synthesized [1-^{14}C]octanoyl coenzyme A was incubated together with DAB and the other ingredients nescessary for the binding reaction of DAB to the enzyme. Fig. 3 shows the radiochromatogram of the products released from the TCA precipitated enzyme protein by mild alkali treatment. A radioactive peak correspond= ing to the area of an authentic sample of OA-DAB (R_f=0.79) was observed. The radioactive peak preceded OA-DAB marker was also found, may be OA judged from its migration.

Fig. 3

The area corresponding to the OA-DAB marker in Fig. 3 was cut out and eluted with 50 % ethanol. The eluate was concentrated and rechromato= graphed with Solvent B (Fig. 4).

Fig. 4

The single peak (R_f=0.81) obtained in the second paper chromatogram was eluted with 50 % ethanol and acid hydrolyzed as described in

"MATERIALS AND METHODS". Fig. 5 shows the radiochromatogram of the hydrolysate of the product. [^{14}C]OA (R_f=0.76) was recovered from the product.

Fig. 5

Incorporation of [1-^{14}C]OA and [4-^{14}C]DAB into OA-DAB — The enzymatically synthesized [1-^{14}C]octanoyl coenzyme A and [4-^{14}C]DAB was incubated as described in "MATERIALS AND METHODS". Fig. 6 shows the radiochromatogram of the products released from the TCA precipitated enzyme protein by mild alkali treatment. A radioactive peak correspond= ing to the OA-DAB marker (R_f=0.79) was observed. The other two peaks which migrated slower than OA-DAB correspomded to DAB and 3-aminopyrro= lidone, respectively. These two compounds were derived from [^{14}C]DAB thioesterfied to the enzyme protein by mild alkali hydrolysis.

Fig. 6

The area corresponding to the OA-DAB marker as indicated by I in Fig 6 was cut out and eluted with 50 % ethanol. The eluate was conden= sed and rechromatographed with solvent B (Fig. 7).

Fig. 7

The single peak (R_f=0.81) obtained in the second paper chromatography was eluted with 50 % ethanol and hydrolyzed as described in "MATERIALS AND METHODS". Fig. 8A and 8B show that [^{14}C]DAB and [^{14}C]OA were recovered from the product. The paper chromatogram was cut out at the area corresponding to DAB and OA, placed in 5 ml of a scintillation fluid, and the radioactivities were determined with a liquid scintillation counter. It was found that the molar ratio of DAB to OA was 1 to 0.7. We consider that the lower recovery of radioactive OA resulted from the presence of endogenous OA in the enzyme preparation as will be discussed later.

Fig. 8

Influence of the Incubation Time on the Enzyme-bound OA-DAB — As shown in Fig. 9, the enzyme-bound OA-DAB was formed linearly for two minutes and then decreased gradually with time. These results suggest that OA-DAB thioesterified to the enzyme protein has a tendency to be libarated from the enzyme under the conditions where no peptide elongation occurred.

Fig. 9

Requirement for the Enzyme-bound OA-DAB Formation — Table II shows that the formation of the enzyme-bound OA-DAB was dependent on

Table II

AS-fraction, Sepharose 4B-fraction and OA-CoA. When the incubation was carried out with AS-fraction alone, OA-DAB synthesis was not observed. On the contrary, in the absence of AS-fraction a small amount of OA-DAB was formed, indicating that Sepharose 4B fraction contains a small quantity of acyltransferase. The last line of the table shows that free OA cannot replace OA-CoA. Thus, several factors which affect the formation of the enzyme-bound OA-DAB were studied.

Effect of the Concentration of AS-fraction on the Enzyme-bound OA-DAB Formation — Fig. 10 shows that AS-fraction is required for the synthesis of the enzyme-bound OA-DAB. A linear relationship was found between the amount of OA-DAB formed and the amount of AS-fraction.

Fig. 10

Effect of the Enzyme Concentration on the Enzyme-bound OA-DAB Formation — The linear relationship between the amount of the enzyme-bound OA-DAB and the amount of the enzyme was shown in a range

up to 200 μg of the enzyme (Fig. 11).

Fig. 11

Effect of OA-CoA Concentration on the Enzyme-bound OA-DAB Formation — The synthesis of the enzyme-bound OA-DAB was dependent on the increasing amount of OA-CoA as shown in Fig. 12. In the absence of OA-CoA, an appropriate amount of the enzyme-bound OA-DAB was formed. This result agrees with the lower recovery of the radioactive OA described in Fig. 8 and may arise from the presence of endogenous OA in the enzyme preparation used in this study. The Km for OA-CoA

Fig. 12

was calculated to be 8×10^{-5}M. The Km values for DL-DAB, L-leucine and L-threonine in the binding reaction to the enzyme were 2.5×10^{-5} M, 6.5×10^{-6} M and 5.0×10^{-6} M, respectively (data not shown).

Incorporation of L-[U-^{14}C]Threonine into the Enzyme-bound Nascent Peptide — When L-[U-^{14}C]threonine was incubated with L-DAB and OA-CoA as described in the legend to Fig. 13, a newly synthesized radioactive peak was found (Fig. 13). However, the radioactive peak

Fig. 13

was not detected in the control incubation without OA-CoA or L-DAB. These results suggest that the acylating reaction is required for the peptide elongation in the biosynthesis of polymyxin E. Although the identity of the radioactive compound is unknown, we consider that it may be a nascent OA-DAB-Thr-DAB-DAB-(DAB) peptide judged from its migration on the paper chromatogram.

DISCUSSION

Because the N-terminal DAB residue is acylated with fatty acids such as MOA, IOA, or OA, the synthesis of the acyl DAB moiety has been the center of the studies in elucidating the biosynthetic mechanism of polymyxin E. Ito *et al.* (19) isolated MOA-DAB and IOA-DAB from a colistin (polymyxin E) producing organism, *Bacillus colistinus*, by feeding [^{14}C]DAB. They also reported that the addition of MOA-DAB or IOA-DAB together with DAB and L-leucine to a reaction mixture consisting of soluble extracts of *B. colistinus* and an ATP generating system enhanced the incorporation of L-[^{14}C]threonine into colistins three times (20). However, the role of MOA-DAB or IOA-DAB is not clear, because a control experiment in which only MOA-DAB or IOA-DAB was omitted was not carried out. We repeated their experiments with crude extracts of *A. polyaerogenes*, but without success. An enzyme activity which catalyzed the ATP-PP_i exchange reaction dependent on IOA-DAB could neither be detected in crude extracts of *A. polyaerogenes*. Thus, it is rather unlikely that free acyl DAB is synthesized first, activated and incorporated into the peptide.

In the gramicidin A synthesizing system, Akashi *et al.* (21-23) presented evidence that the N-terminal amino acid, valine, bound to its activating enzyme was formylated, and then peptide elongation took place via the formation of formylvalylglycine. Mohr and Kleinkauf (24) also suggested that the biosynthesis of alamethicin was initiated by acetylation of aminoisobutyric acid thioesterified to the enzyme. Zocher and Kleinkauf (25) showed that methylation of enzyme-bound valine occurred during the synthesis of enniatin B. As described in this communication, OA-DAB thioesterified to the enzyme

was synthesized by the DAB activating enzyme and acyltransferase fraction (AS-fraction) prepared from polymyxin E producing organism. At present it is not completely resolved whether the OA-DAB bound to the enzyme truely participates in the initiation of polymyxin E biosynthesis or not. However, the incubation of [^{14}C]threonine together with L-DAB and the other ingredients except OA-CoA did not give rise to any enzyme-bound intermediary peptides, while in the presence of the acylating system an nascent peptide thioesterified to the enzyme was formed (Fig. 13). This preliminary evidence supports a hypothesis that the initiation of polymyxin E synthesis occurs with the acylation of DAB thioesterified to the enzyme protein.

The isolation and identification of enzyme-bound peptides are being studied.

REFERENCES

1. Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445-459
2. Kurahashi, K. in Antibiotics. Biosynthesis (Corcoran, J.W., ed.) Vol. IV, Springer-Verlag, Berlin·Heidelberg·New York. in press
3. Laland, S.G. & Zimmer, T.L. (1973) in Essays in Biochemistry (Campbell, P.N. & Greville, G.D. eds) Vol. 9, pp 31-57, Academic Press, London
4. Lipmann, F. (1971) Science 173, 875-884
5. Kleinkauf, H. & Koischwitz, H. (1978) Prog. Mol. Subcellular Biol. 6, 59-112
6. Katz, E. & Demain, A.L. (1977) Bacteriol. Rev. 41, 449-474
7. Suzuki, T., Hayashi, K., Fujikawa, K., & Tsukamoto, K. (1965) J. Biochem. 57, 226-227
8. Koyama, Y., Kurosawa, A., Tuchida, A., & Takahashi, K. (1950) J. Biochem. 3, 457
9. Komura, S. & Kurahashi, K. (1979) J. Biochem. 86, 1013-1021
10. Withander, L. & Heding, H. (1976) J. Antibiotics 29, 774-775
11. Ciferri, O., DiGirolamo, M., & DiGirolamo, A.B. (1961) Nature 191,411
12. Brenner, M., Gray, E., & Paulus, H. (1964) Biochim. Biophys. Acta 185, 401-403
13. Jayaraman, K., Monreal, J., & Paulus, H. (1969) Biochim. Biophys. Acta 185, 447-457
14. Komura, S. & Kurahashi, K. J. Biochem. (1980) in press
15. Bar-Tana, J., Rose, G., & Shapiro, B. (1975) Methods in Enzymology (Lowenstein, J.M., ed.) Vol. XXXV, pp 117-122, Academic Press, New York

16. Pardee, A.B., Jacob, F., & Monod, J. (1959) J. Mol. Biol. 1 165

17. Chance, B. & Maehly, A.C. (1955) Methods in Enzymology (Colowick, S.P. & Kaplan, N.O., eds.) Vol. II, pp 764-775, Academic Press, New York

18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

19. Ito, M., Aida, K., & Uemura, T. (1970) Agr. Biol. Chem. 34, 476-479

20. Ito, M., Koyama, Y., Aida, K., & Uemura, T. (1970) Biochim. Biophys. Acta 215, 418

21. Akashi, K. & Kurahasi, K. (1977) Biochem. Biophys. Res. Commun. 77, 259-267

22. Akashi, K., Kubota, K., & Kurahashi, K. (1977) J. Biochem. 81, 269-272

23. Akashi, K. & Kurahashi, K. (1978) J. Biochem. 83, 1219-1229

24. Mohr, H. & Kleinkauf, H. (1978) Biochim. Biophys. Acta 526 375-386

25. Zocher, R. & Kleinkauf, H. (1978) Biochem. Biophys. Res. Commun. 81, 1162-1167

Table I. Purification of the L-DAB activating enzyme. The enzyme was pufiried from 54 g cells as described in "MATERIALS AND METHODS".

Fraction	Protein (mg)	Specific activity[a]	Yield (%)
1. Crude extract	3,440	0.37	100
2. 78,000 x g supernatant	2.760	0.42	91
3. As-fraction	805	1.22	77
4. Sepharose 4B-fraction	54	8.66	37

[a] Units/mg of protein; one unit of activity is defined as that amount of enzyme which exchanges 1 µmole of PP_i with ATP in 30 min under the conditions described in "MATERIALS AND METHODS".

Table II. Requirement for the enzyme-bound OA-DAB formation. Incubation was carried out as described in "MATERIALS AND METHODS" except for the omissions or additions as indicated. In this particular experiment, the Sepharose 4B fraction of the second column chromatography as described in a previous paper (14) was used. 600 µg of Sepharose 4B fraction and 124 µg of AS-fraction were used.

Conditions	[^{14}C]DAB incorporated into OA-DAB (cpm)
Complete	228
- Incubation	0
- AS-fraction	14
- Sepharose 4B fraction	72
- OA-CoA	0
- OA-CoA, + OA	0

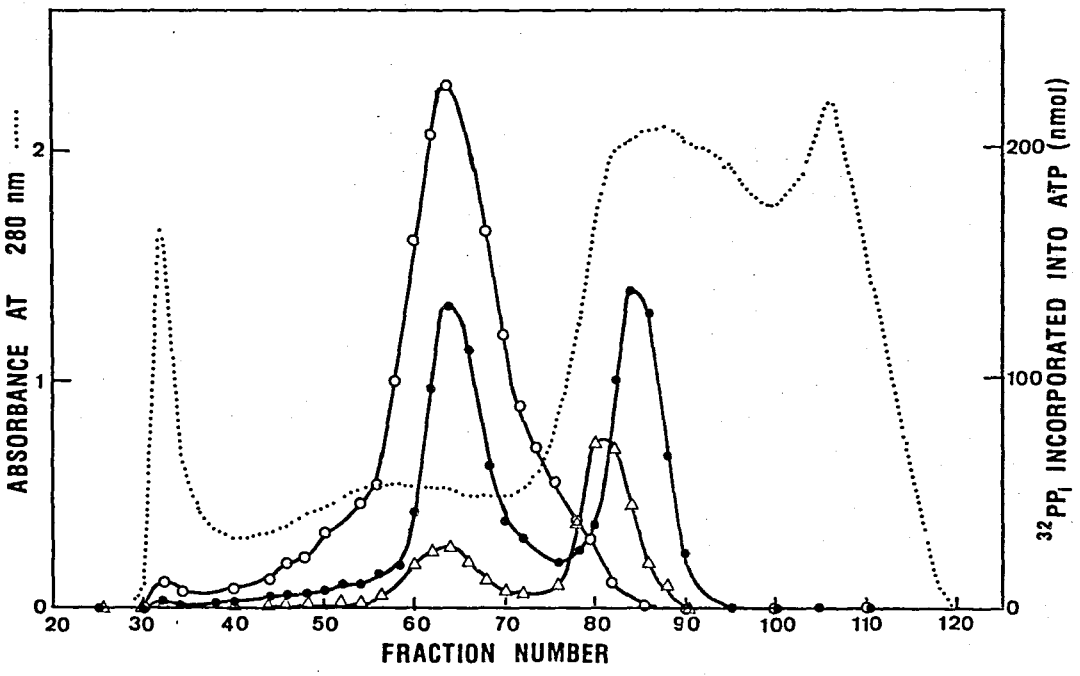


Fig. 1. Gel filtration of the L-DAB activating enzyme on a Sepharose 4B column. The enzyme protein was eluted as described in "MATERIALS AND METHODS". A sample of 50 µl of each fraction was assayed for ATP-PP_i exchange activity. -----, Absorbance at 280 nm; -o-, L-DAB dependent exchange activity; -●-, L-leucine dependent exchange activity; -Δ-, L-threonine dependent exchange activity.

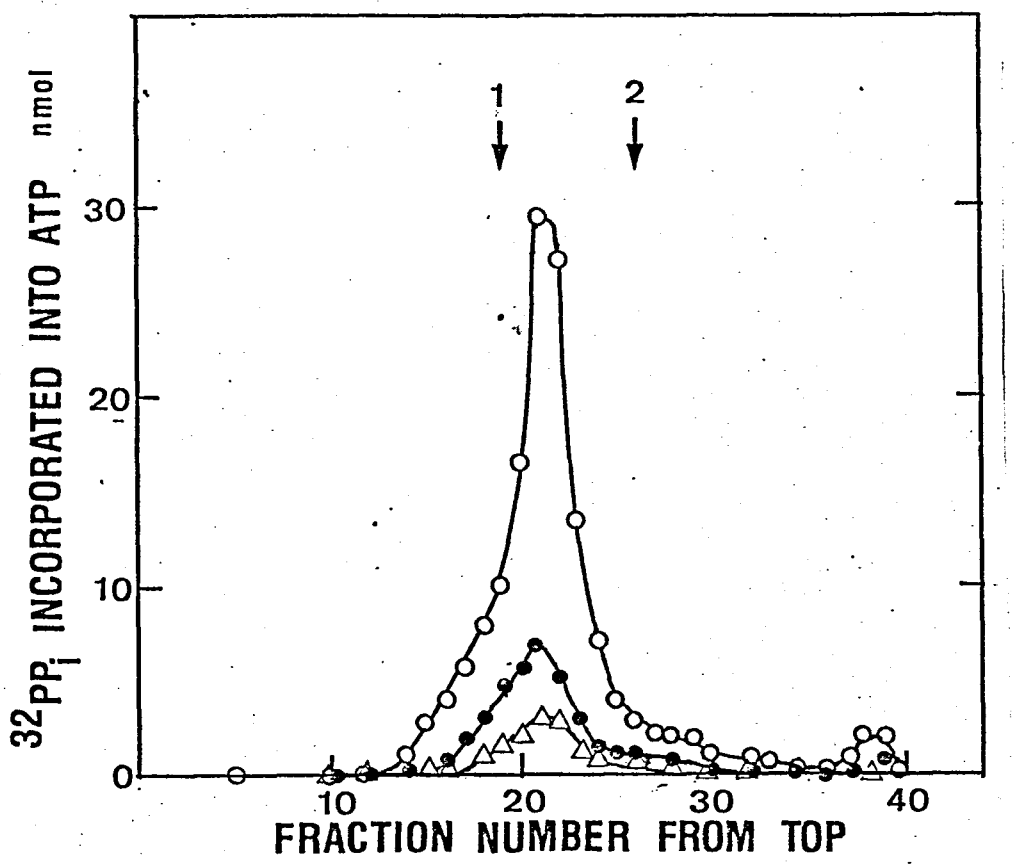


Fig. 2. Sucrose density gradient centrifugation of the L-DAB activating enzyme. The procedures were described in "MATERIALS AND METHODS". 50 µl of each fraction was assayed for ATP-PP_i exchange activity dependent on the amino acid indicated. - o -, L-DAB; - • -, L-leucine, - Δ -, L-threonine.

Arrows 1 and 2 indicate the positions of catalase (11.3 S) and β-galactosidase (15.9 S), respectively.

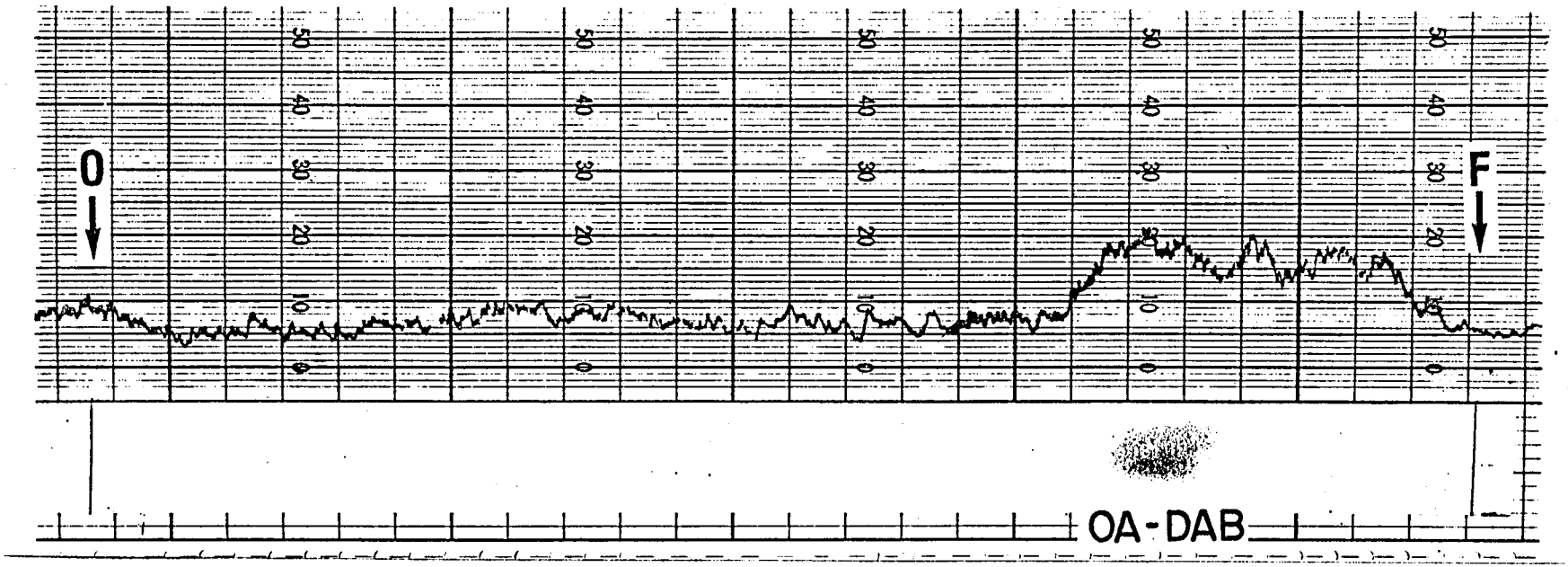


Fig. 3. Paper chromatography of the [^{14}C]OA-labelled products thioesterified to the enzyme. The reaction was carried out as described in "MATERIALS AND METHODS" except that 40 nmol of [1-^{14}C]OA-CoA and 200 nmol of L-DAB were used. 624 µg of Sepharose 4B fraction and 206 µg of AS-fraction were used. Ten identical reaction mixtures were incubated and combined for analysis by descending paper chromatography with a Toyo No. 50 filter paper. Solvent A was used. The radioactivity was scanned with a Packard radiochromatogram scanner Model 7201. The OA-DAB marker was visualyzed by ninhydrin spray.

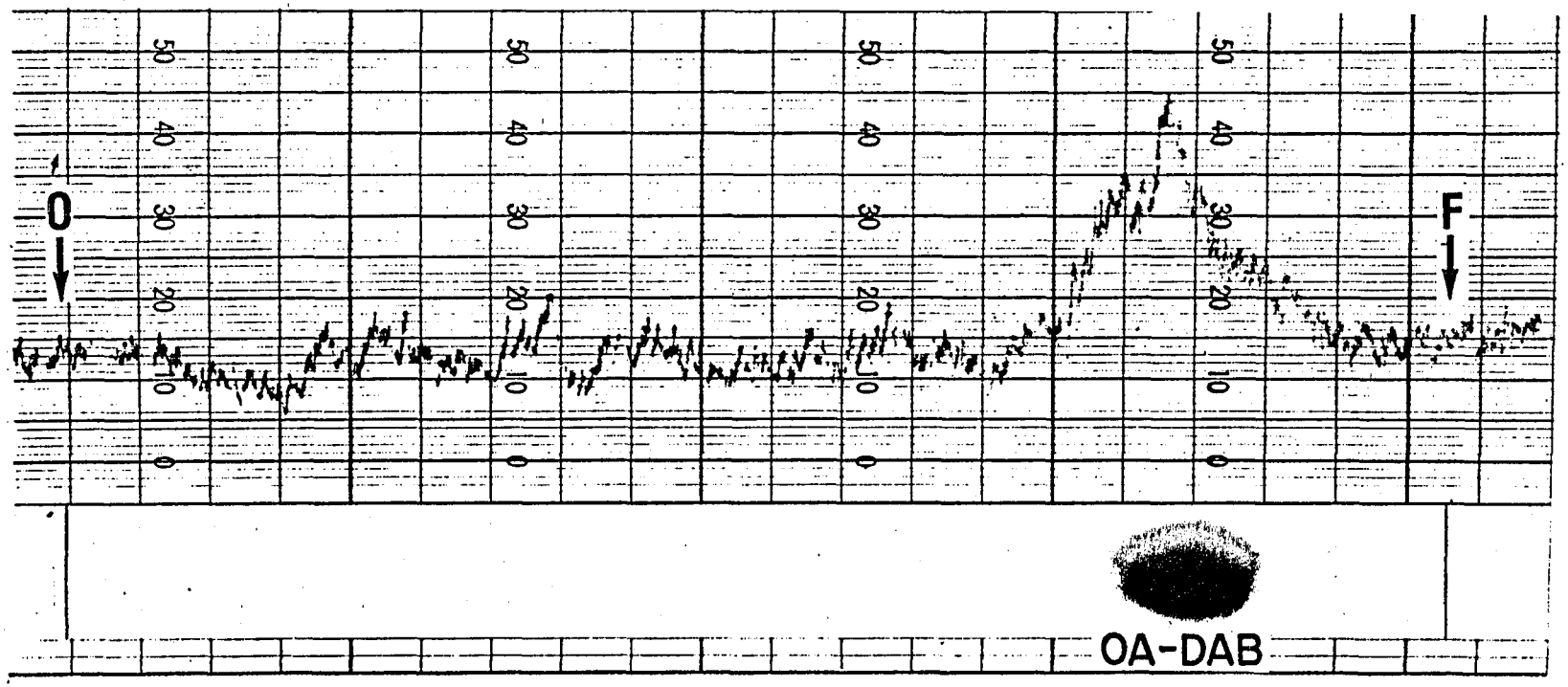


Fig. 4. The second paper chromatotraphy of the product labelled with [^{14}C]OA comigrating with OA-DAB. Paper chromatography was carried out descendingly on a Toyo No. 51A filter paper with Solvent B.

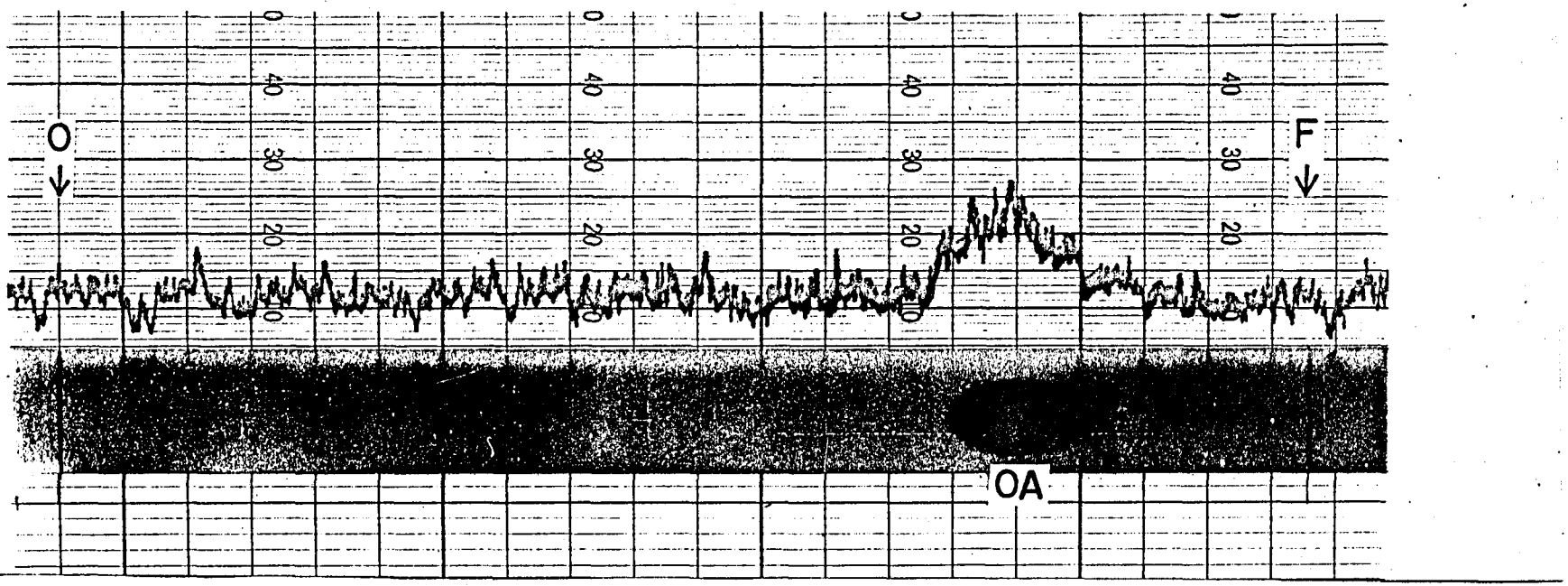


Fig. 5. Paper chromatography of acid hydrolyzate of the OA-DAB fraction labelled with [^{14}C]OA. The ethereal fraction of the hydrolyzate prepared as described in "MATERIALS AND METHODS" was chromatographed ascendingly on a Toyo No. 50 filter paper with Solvent C. The OA marker was detected by bromocresol green spray.

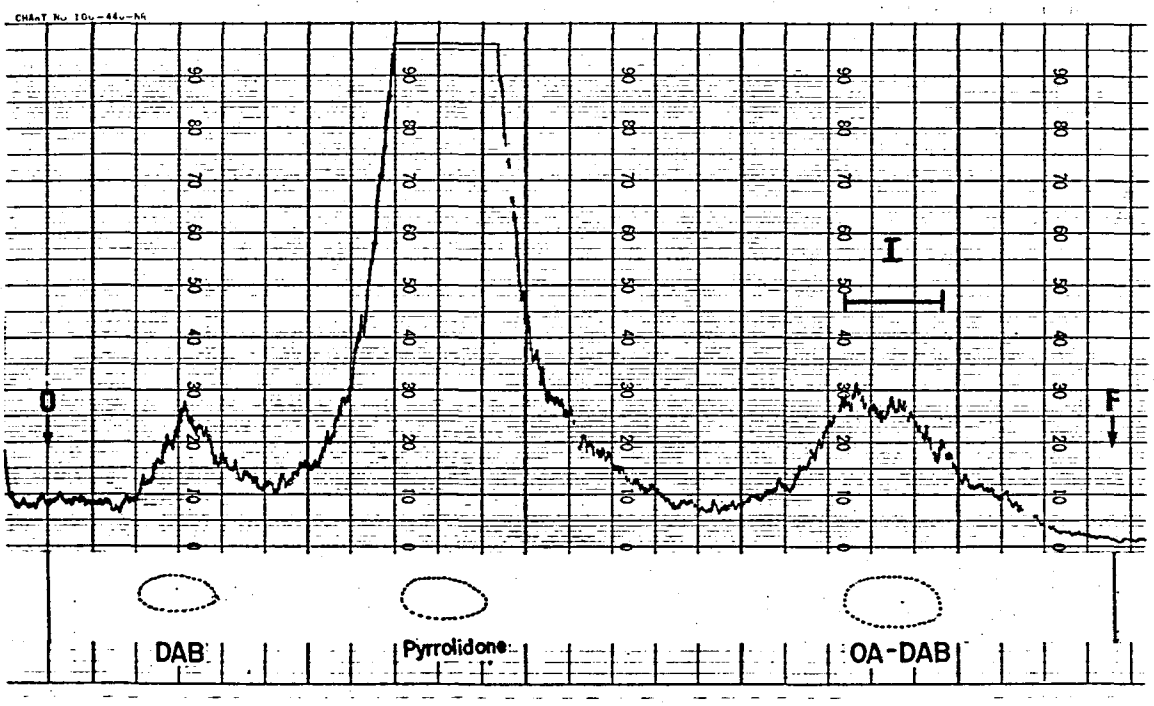


Fig. 6. Paper chromatography of the [^{14}C]OA and [^{14}C]DAB labelled products thioesterified to the enzyme. The reaction was carried out as described in "MATERIALS AND METHODS" except that 40 nmol of [1-^{14}C]OA-CoA and 9 nmol of DL-[4-^{14}C]DAB were used. 624 µg of Sepharose 4B fraction and 206 µg of AS-fraction were used. Ten identical reaction mixtures were incubated and combined for analysis by descending paper chromatography with a Toyo No. 50 filter paper. Solvent A was used. The radioactivity was scanned with the Packard radiochromatogram scanner.

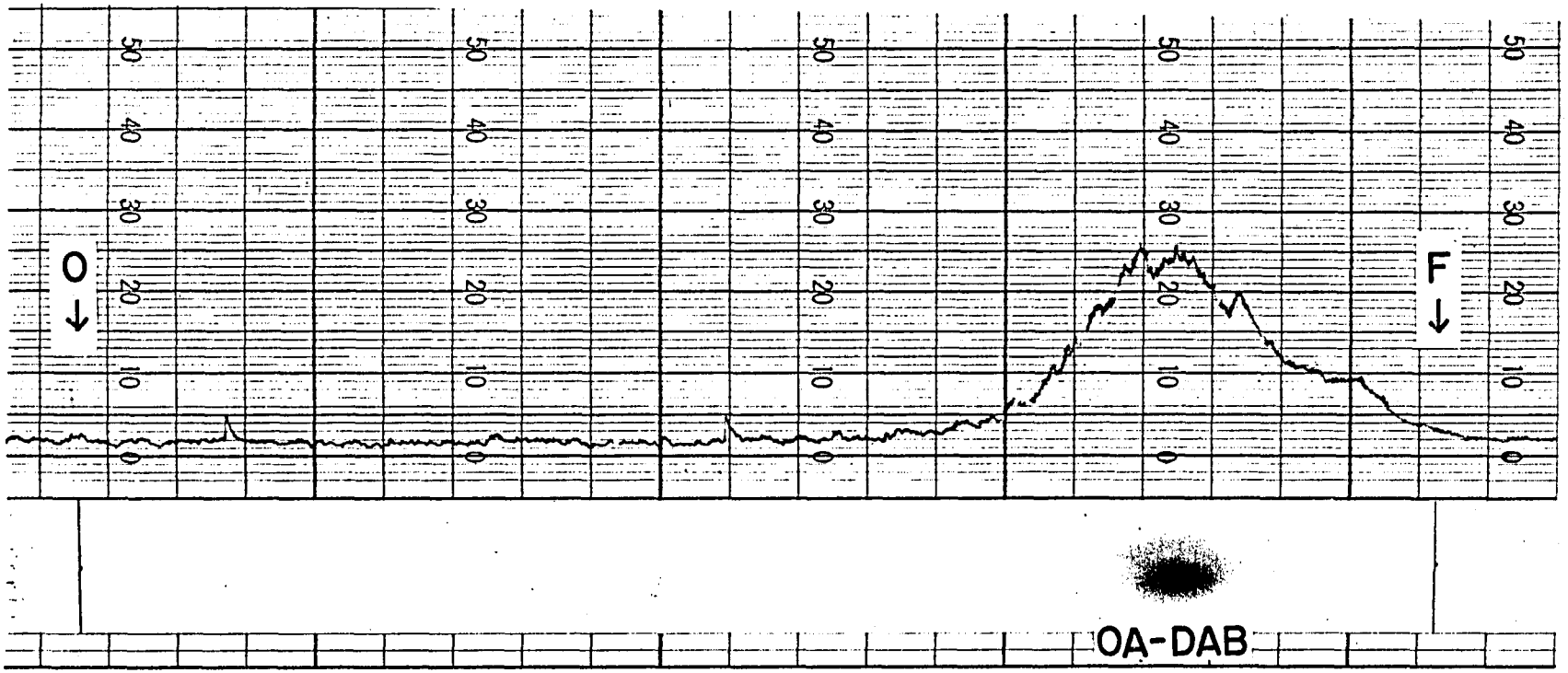


Fig. 7. The second paper chromatography of the product labelled with [^{14}C]OA and [^{14}C]DAB comigrating with OA-DAB. Paper chromatography was carried out descendingly on a Toyo No. 51A filter paper with Solvent B.

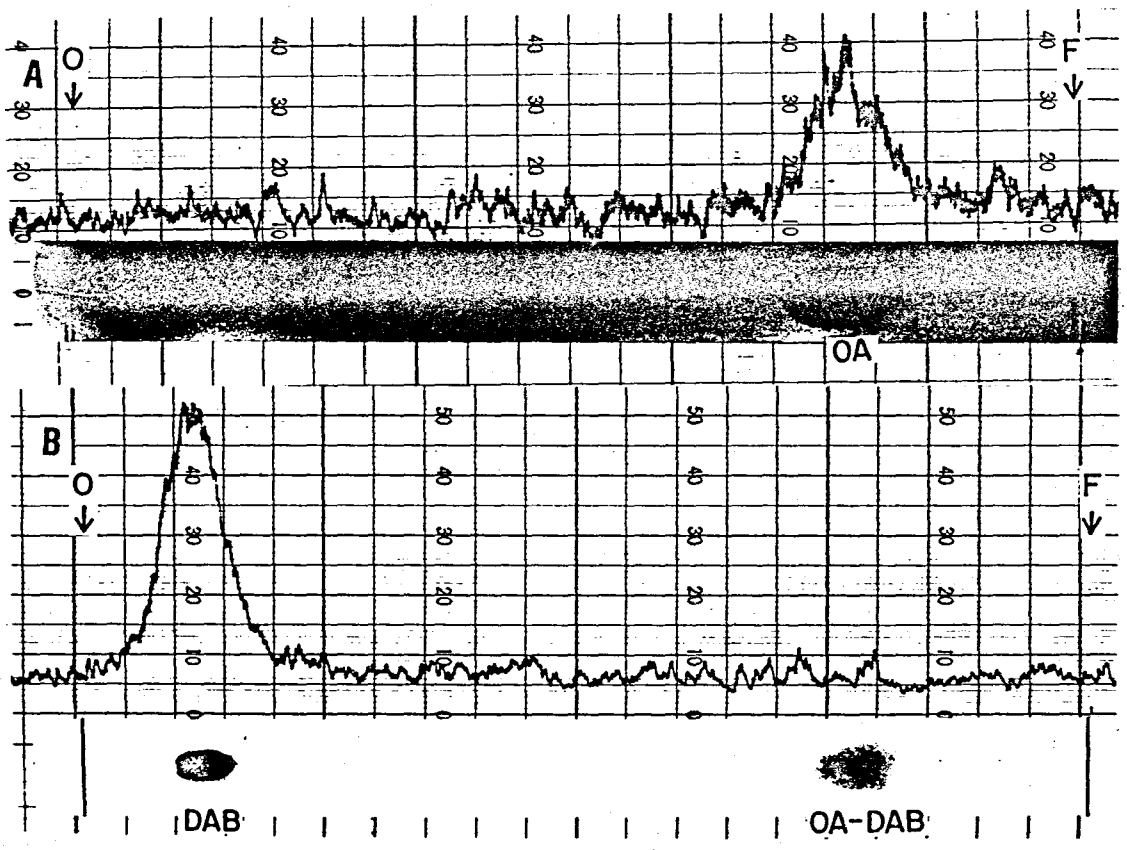


Fig. 8. Paper chromatography of the acid hydrolyzate of the OA-DAB fraction labelled with [^{14}C]OA and [^{14}C]DAB. (A) The ethereal fraction of the hydrolyzate was chromatographed as described in the legend to Fig. 5. (B) The aqueous fraction of the hydrolyzate was chromatographed descendingly on a Toyo No. 50 filter paper with Solvent A.

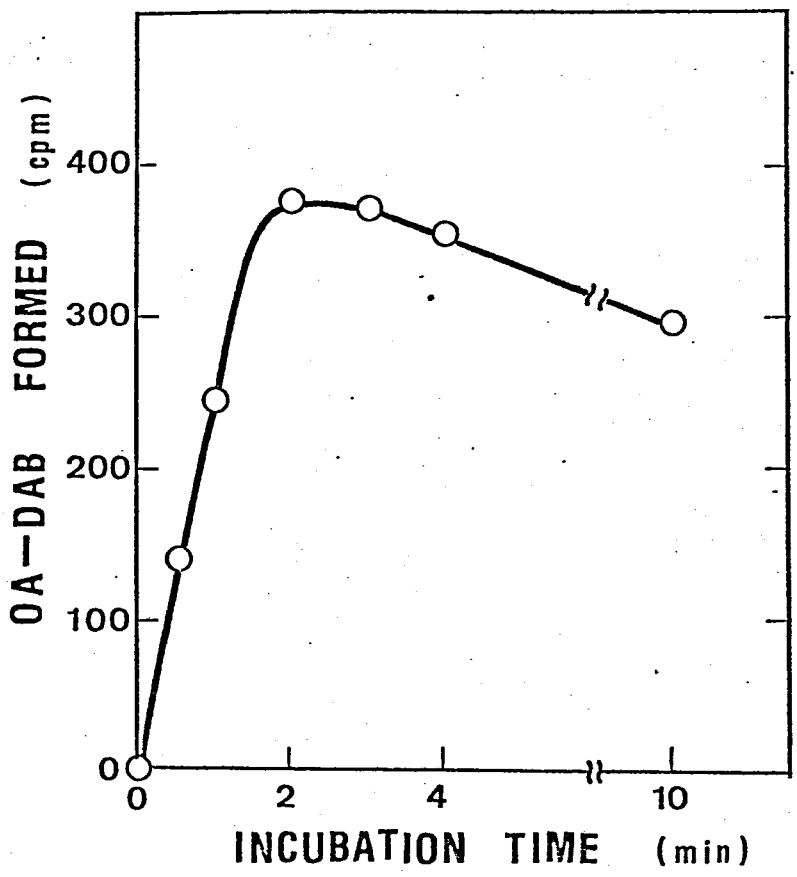


Fig. 9. Influence of the incubation time on the enzyme-bound OA-DAB formation. Experimental procedures were described in "MATERIALS AND METHODS"; 624 µg of Sepharose 4B fraction and 206 µg of AS-fraction were used.

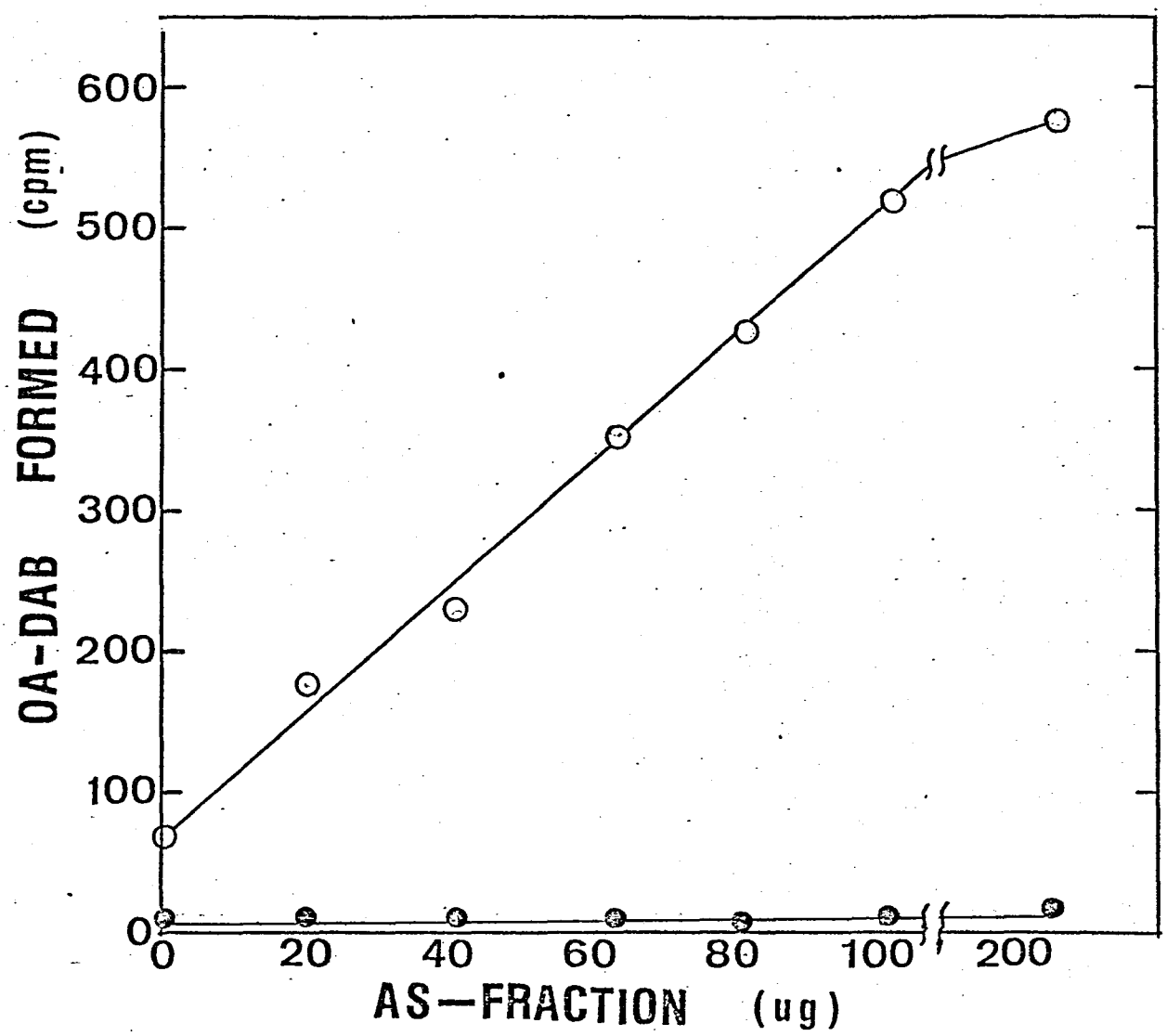


Fig. 10. Effect of the concentration of AS-fraction on the enzyme-bound DAB formation. Experimental procedures were described in "MATERIALS AND METHODS"; 208 µg of Sepharose 4B fraction was used.
-o-, Incubation with Sepharose 4B fraction; -●-, incubation without Spharose 4B fraction.

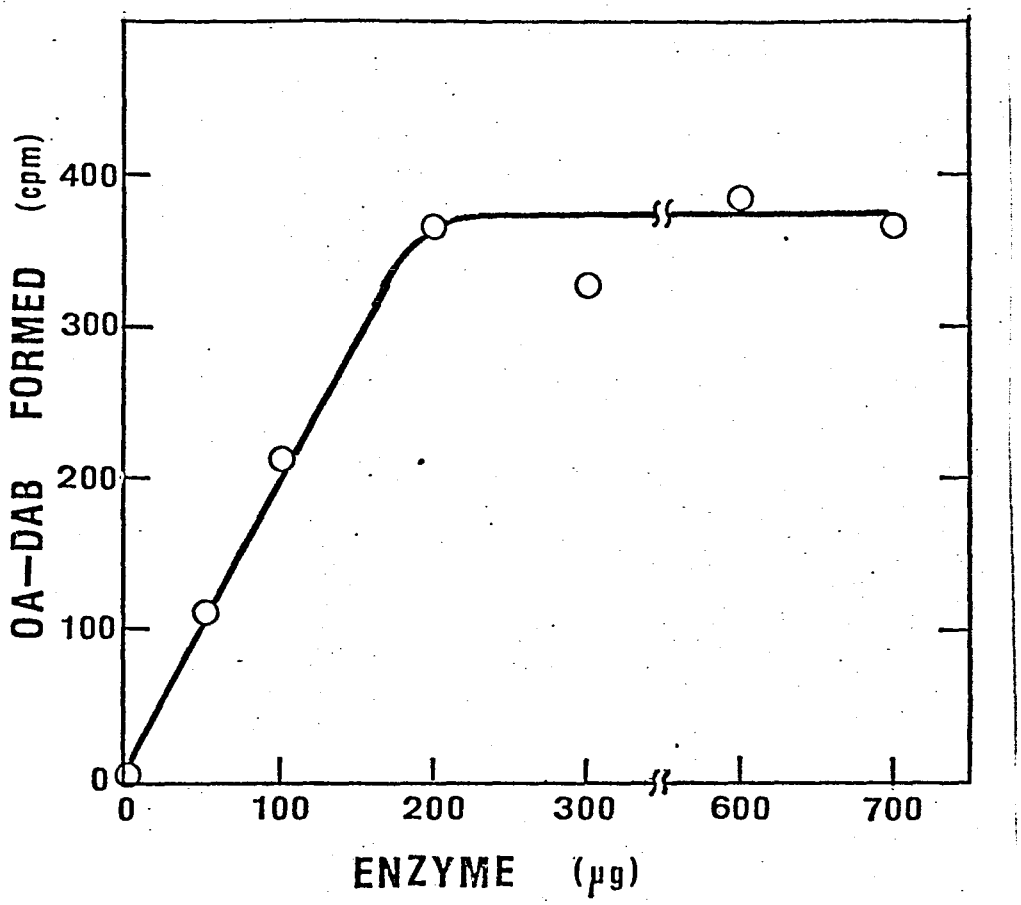


Fig. 11. Effect of the enzyme concentration on the enzyme-bound OA-DAB formation. Experimental procedures were described in "MATERIALS AND METHODS"; 206 μg of AS-fraction was used.

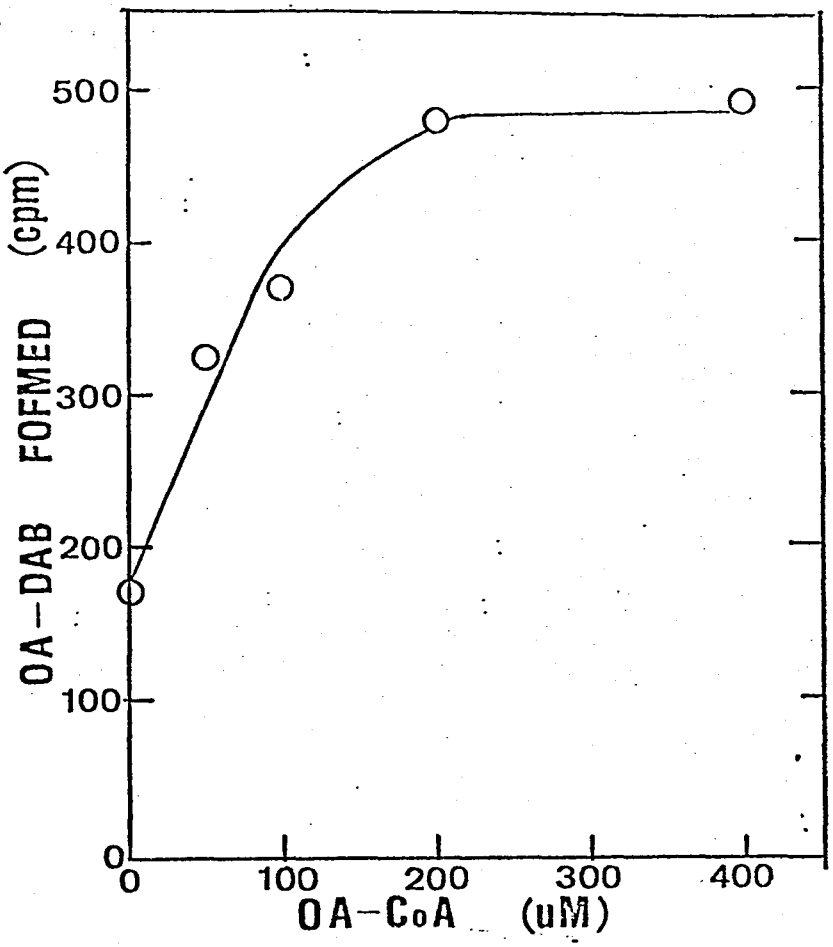


Fig. 12. Effect of the octanoyl coenzyme A concentration on the enzyme-bound OA-DAB formation. Experimental procedures were described in "MATERIALS AND METHODS"; 624 µg of Sepharose 4B fraction and 206 µg of AS-fraction were used.

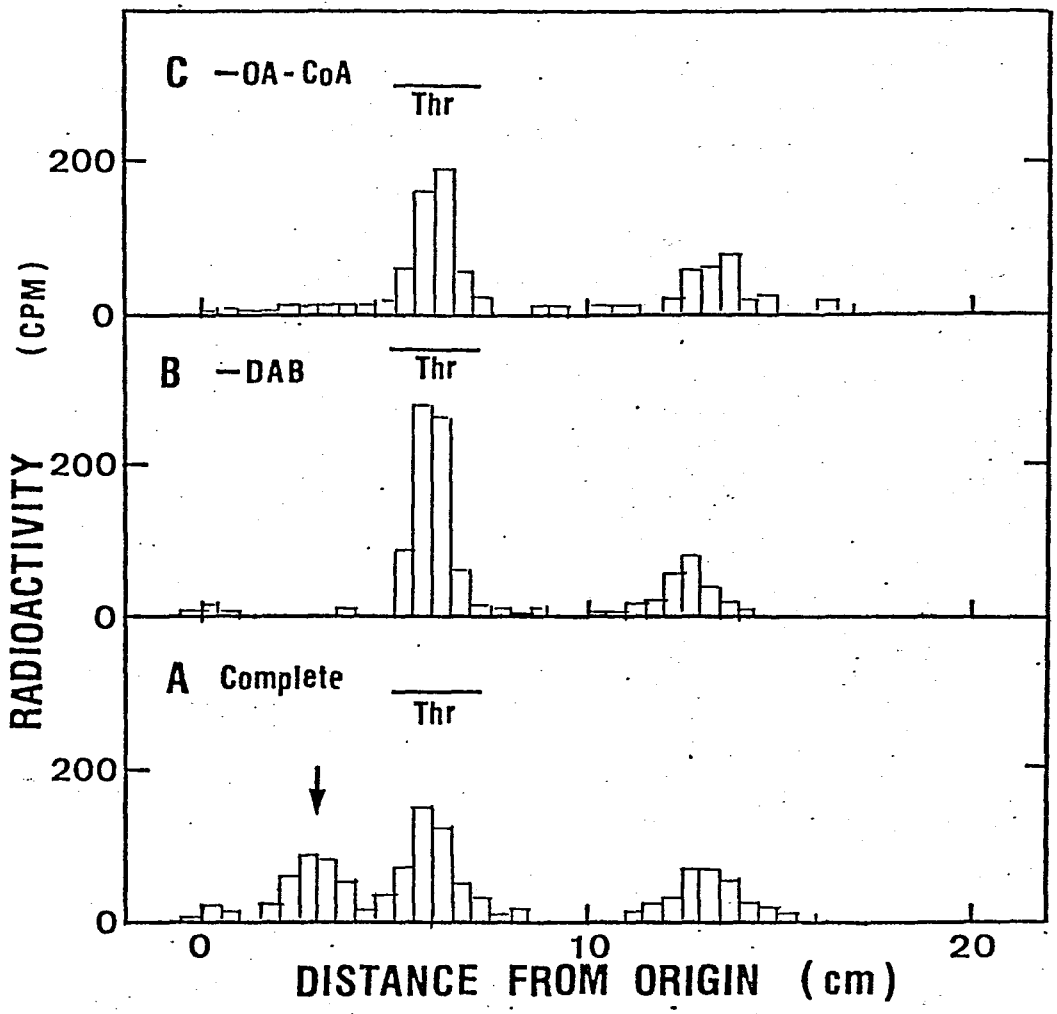


Fig. 13. Incorporation of L-[^{14}C]threonine into nascent peptides. The incubation mixture was the same as that of the OA-DAB formation described in "MATERIALS AND METHODS" except that 8 nmol of L-[U-^{14}C]-threonine and 200 nmol of cold L-DAB were used. 900 µg of Sepharose 4B fraction and 216 µg of AS-fraction were used.

PART III

Total Synthesis of Polymyxin E by a Cell-free Enzyme System

Abbreviations : DAB, 2,4-diaminobutyric acid; OA, octanoic acid; OA-CoA, octanoyl coenzyme A.

SUMMARY

Polymyxin E, an antimicrobial branched cyclic decapeptide, was synthesized by an enzyme fraction partially purified from crude extracts of the producing organism, Aerobacillus polyaerogenes. For the synthesis, three constituent amino acids (L-2,4-diaminobutyric acid, L-leucine, and L-threonine), ATP, Mg^{2+} and an acylating system consisting of octanoyl CoA and an ammonium sulfate fraction of cell extracts are required.

Polymyxin E (colistin) produced by Aerobacillus polyaerogenes or Bacillus colistinus Koyama is one of the polymyxin series of antibiotics effective against gram negative bacteria (1, 2). The amino terminal residue, L-2,4-diaminobutyric acid (DAB), is acylated with either 6-methyloctanoic acid, isooctanoic acid, or octanoic acid (OA) as shown in Diagram I (3).

Studies of the biosynthesis of antibiotic peptides produced by bacteria of the genus Bacillus, such as gramicidin S, tyrocidines, bacitracins and gramicidin A, revealed that they were synthesized by the multienzyme thiotemplate mechanism without the involvement of nucleic acids or ribosomes (4-10). A number of studies using whole cells showed that inhibitors of protein synthesis had no effect on polymyxin production (11-13), suggesting that its biosynthesis was carried out according to the multienzyme thiotemplate mechanism. Ito et al. (14) reported that crude extracts of B. colistinus Koyama

catalyzed the synthesis of colistin (polymyxin E), but the details of the biosynthetis mechanism have remained unclarified.

We reported previously that a partially purified enzyme of A. polyaerogenes could activate and bind DAB, L-threonine and L-leucine through thioester bonds (2). We further proved that OA was transferred from octanoyl coenzyme A (OA-CoA) to a part of enzyme-bound DAB, when an ammonium sulfate fraction of the crude extracts was added as a source of acyltransferase to the reaction for the amino acid binding experiments (15). In this communication we present evidence that the same enzyme system is able to synthesize polymyxin E when the three constituent amino acids of polymyxin E are present in the reaction mixture.

MATERIALS AND METHODS

Chemicals and enzymes. The following chemicals were obtained commercially : L-DAB, OA-CoA and phosphoenolpyruvate from Sigama Chemical Co., L-[U-^{14}C]leucine (240 mC$_i$/mmole) from Daiichi Pure Chemical Co. Inorganic pyrophosphatase [EC 3.6.1.1] and pyruvate kinase [EC 2.7.1.40] were purchased from Boehringer Mannheim GmbH. Polymyxin E sulfate and deacylpolymyxin E were the generous gift from Banyu Pharmaceutical Co. Ltd. and Dr. Y. Kimura, respectively.

Enzyme preparation. The preparation of the DAB activating enzyme (Sepharose 4B fraction) and an ammonium sulfate fraction were described in earlier papers (2, 14).

Incubation for polymyxin E biosynthesis. The reaction mixture contained, in a final volume of 0.2 ml, 10 µmoles of Tris-HCl buffer (pH 7.7), 0.8 µmole of ATP, 2 µmoles of $MgCl_2$, 1 µmole of dithiothreitol, 0.8 µmole of phosphoenolpyruvate, 0.2 µmole of L-DAB, 0.2 µmole of L-threonine, 0.16 µmole of OA-CoA, 4 nmoles of L-[U-^{14}C]leucine (372,800 cpm), 206 µg of the ammonium sulfate fraction and 312 µg of the enzyme (Sepharose 4B fraction). The mixture was incubated at 30°C for 40 minutes. After incubation, the reaction was stopped by addition of 10 µl of trichloroacetic acid to give a final concentration of 4.8 %. Fifty micrograms of polymyxin E sulfate and 1 µmole of L-leucine were then added as carriers. After standing at 0°C for 1 hour, the precipitated protein was removed by centrifugation. The pH of the supernatant solution was adjusted to 7 by addition of 1 N KOH. The methods of isolation and

analysis of the products are described in the legends to Fig. 1 and 3.

Paper chromatography. Paperchromatography was carried out descendingly on Toyo No. 50 filter paper with the following solvent systems.

Solvent A : n-butanol : acetic acid : pyridine : H_2O
(15 : 3 : 10 : 12)

Solvent B : n-butanol : acetic acid : H_2O
(4 : 1 : 2)

RESULTS

Incorporation of L-[^{14}C]leucine into polymyxin E. The enzyme fraction was incubated with L-[^{14}C]leucine, DAB, L-threonine together with ATP and the DAB acylating system as described in MATERIALS AND METHODS. After incubation, the reaction was stopped and the supernatant solution was applied to a column of Bio-Gel P-2. Fig. 1 shows the gel-filtration pattern of the radioactive products. The radioactive peak preceding the large free [^{14}C]leucine peak coincided with the position where polymyxin E was eluted. Fractions 8 throgh 10 were condensed and subjected to paper chromatography with two different solvent systems as shown in Fig. 2. The R_f of the radioactive compound was the same as that of an authentic sample of polymyxin E. [^{14}C]Leucine incorporated into the polymyxin E fraction accounted for 9.8 % of the total input in the reaction

Requirement for the total synthesis of polymyxin E. Polymyxin E is a strongly basic peptide because it possesses six residues of DAB. It adsorbs to Dowex 50 (NH_4^+-form) column very tightly and can be eluted only with a very basic solvent such as 2 N piperidine (Hayashi, K., personal communication). A reaction for polymyxin E synthesis was carried out as described in MATERIALS AND METHODS and the radioactive products were chromatographed on a Dowex 50W-X4 column. Fig. 3 shows

the elution pattern of the radioactive products. The control without incubation or without OA-CoA, DAB and threonine shows that the radioactive product, which was eluted with 2 N piperidine, was not formed. The radioactive product eluted with 2 N piperidine was verified to be polymyxin E by paper chromatography (data not shown).

Table I shows the requirement for the incorporation of L-[^{14}C]leucine into polymyxin E. Each of Sepharose 4 B fraction, DAB, threonine, OA-CoA and an ammonium sulfate fraction is essential for the total synthesis of polymyxin E by the enzyme system. In the absence of threonine and an ammonium sulfate fraction, there was an appreciable amount of incorporation of [^{14}C]leucine; we think that it resulted from the presence of endogenous threonine in the enzyme preparations and from the presence of acyltransferase contaminating in Sepharose 4B fraction, respestively.

DISCUSSION

The enzyme protein which catalyzed the total synthesis of polymyxin E had an approximate molecular weight of 300,000 (15). It activates and binds DAB, L-leucine and L-threonine as thioesters. About one tenth of DAB thioesterified to the enzyme is acylated by transacylase and OA-CoA (15). The addition of the acylating system is essential for the total synthesis of polymyxin E (Table 1). When DAB and [^{14}C]threonine were incubated under the conditions of the binding experiments without the acylating system, no intermediary peptide bound to the enzyme was formed (unpublished results). These results suggest that the synthesis of polymyxin E takes place with the initial

formation of the enzyme-bound OA-DAB followed by peptidation to a decapeptides stage and cyclization. It was reported that the initiation of gramicidin A biosynthesis was the formation of formyl-valine thioesterified to the synthetase (16-18). Lipmann and his group reported that the large enzyme of tyrocidine synthetase with a molecular weight of 440,000 consist of six 70,000 dalton-subunits which activate six each constituent amino acid (19, 20). It is rather unexpected that polymyxin synthetase with a molecular weight of 300,000 can activate, thioesterify and conjugate ten constituent amino acids. No attempts to demonstrate enzyme-bound phosphopantetheine has been made. We are trying to purify further the synthetase and elucidate the mechanism of the total synthesis of polymyxin E.

ACKNOWLEDGEMENTS

We are grateful to Dr. K. Hayashi of Kyoto University for supplying us the bacterial strain and authentic sample of polymyxin E_3. We thank Dr. Y. Kimura of Mukogawa Women's University and Mr. H. Takeda of Banyu Pharmaceutical Co. Ltd. for the generous gift of deacylated polymyxin E and polymyxin E, respectively.

REFERENCES

1. Suzuki, T., Hayashi, K., Fujikawa, K., and Tsukamoto, K. (1965) J. Biochem. 57, 226-227
2. Komura, S., and Kurahashi, K. (1979) J. Biochem. 86, 1013-1021
3. Withander, L., and Heding, H. (1976) J. Antibiotics 29, 774-775
4. Kurahashi, K., Yamada, M., Mori, K., Fujikawa, K., Kambe, M., Imae, Y., Sato, E., Takahashi, H., and Sakamoto, Y. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 815-826
5. Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445-459
6. Kurahashi, K. in Antibiotics. Biosynthesis (Corcoran, J. W., ed.) Vol. IV, Springer-Verlag, Berlin-Heidelberg-New York, in press
7. Lipmann, F., Gevers, W., Kleinkauf, H., and Roskoski, R., Jr. (1971) Advan. Enzymol. 33, 337-380
8. Lipmann, F. (1971) Science 173, 875-884
9. Laland, S. G., and Zimmer, T. L. (1973) in Essays in Biochemistry (Campbell, P. N., and Greville, G. D., eds.) Vol. 9, pp. 31-57 Academic Press, London
10. Frøyshov, Ø., Zimmer, T. L., and Laland, S. G. (1978) Int. Rev. Biochem. 18, 49-78
11. Daniels, M. J. (1968) Biochim. Biophys. Acta 156, 119-127
12. Monreal, J., and Paulus, H. (1970) Biochi. Biophys. Acta 199, 280-282
13. Ito, M., Aida, K., and Uemura, T. (1970) Biochim. Biophys. Acta 213, 244-247
14. Ito, M., Koyama, Y., Aida, K., and Uemura, T. (1970) Biochim. Biophys. Acta 215, 418-420
15. Komura, S., and Kurahashi, K. (1980) J. Biochem. in press
16. Akashi, K., Kubota, K., and Kurahashi, K. (1977) J. Biochem. 81, 269-272
17. Akashi, K., and Kurahashi, K. (1977) Biochem. Biophys. Res. Commun. 71, 259-267
18. Akashi, K., and Kurahashi, K. (1978) J. Biochem. 83, 1219-1229
19. Lee, S. G., Roskoski, R., Jr., Bauer, K., and Lipmann, F. (1973) Biochemistry 12, 398-405
20. Lee, S. G., and Lipmann, F. (1977) Proc. Natl. Acad. Sci. U. S. 74, 2343-2347

Table 1. Requirement for the Incorporation of L-[^{14}C]Leucine into polymyxin E.

Conditions	Radioactivity incorporated into polymyxin E (cpm)
Complete	7,873
- DAB	113
- threonine	1,573
- octanoyl coenzyme A	193
- ammonium sulfate fraction	2,240

The reaction and isolation of the supernatant solution were carried out as described in MATERIALS AND METHODS. The supernatant solution was applied to a Dowex 50w-X4 (NH_4^+-form) column (1 X 1 cm), and then the column was washed as described in the legend to Fig. 3. The effluent with 2 N piperidine was evaporated to dryness and subjected to paper chromatography with Solvent B. The radioactivity in the area corresponding to the marker of authentic polymyxin E was determined with a Beckman liquid scintillation counter.

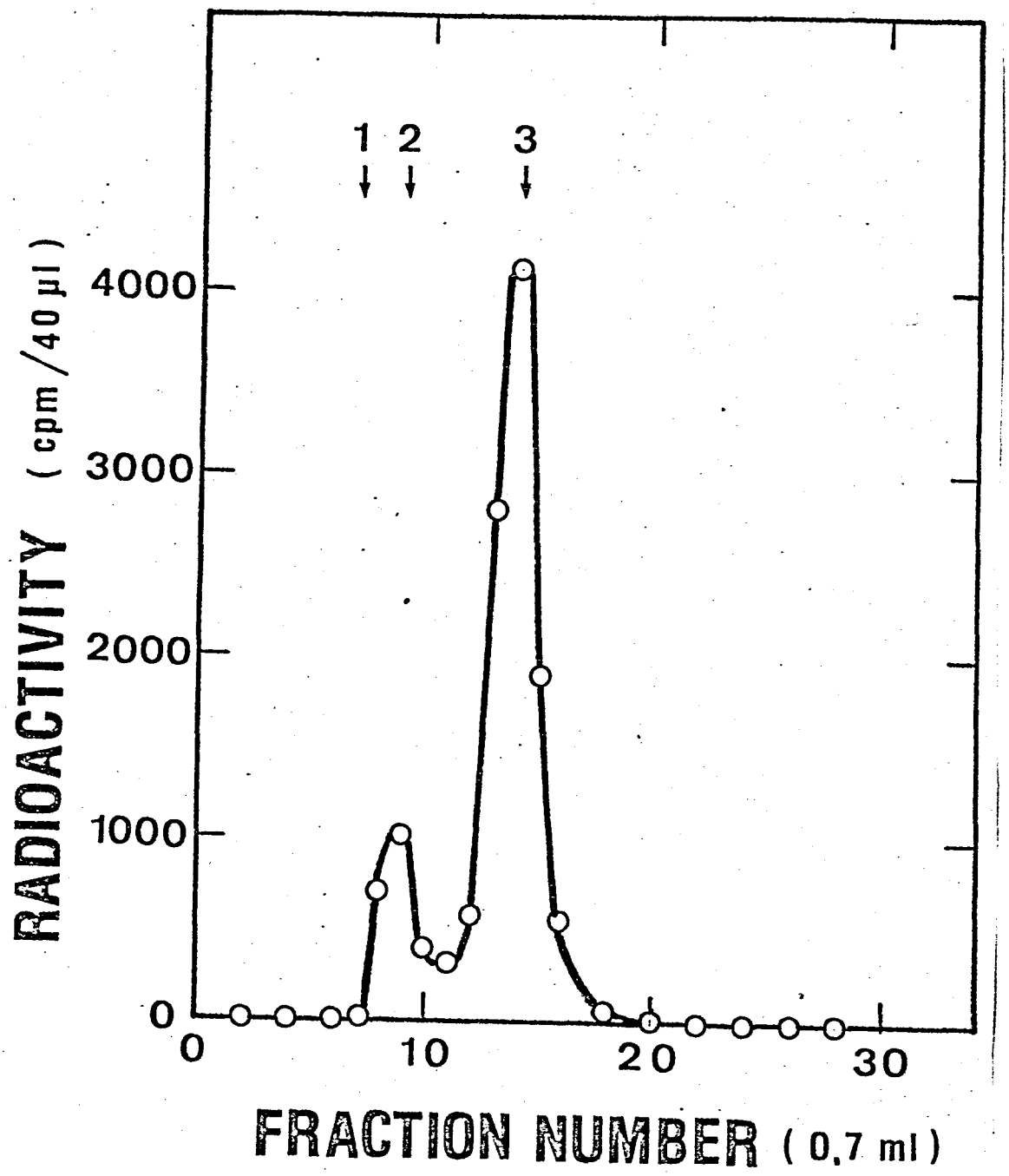


Fig. 1. Gel Filtration of the Reaction Products on a Bio-Gel P-2 Column. The supernatant solution of the reaction mixture was obtained as described in MATERIALS AND METHODS and applied to a Bio-Gel P-2 column (1 x 20 cm) previously equilibrated with 0.2 N acetic acid. The column was eluted with 0.2 N acetic acid. Forty microliters of each fraction were applied to a Whatman GF/C glass fiber paper. The paper was dried, placed in 5 ml of a scintillation fluid and the radioactivity was determined with a Beckman Model LS-250 liquid scintillation counter. Arrows 1, 2 and 3 indicate the elution position of blue dextran 2000, polymyxin E and L-leucine, respectively.

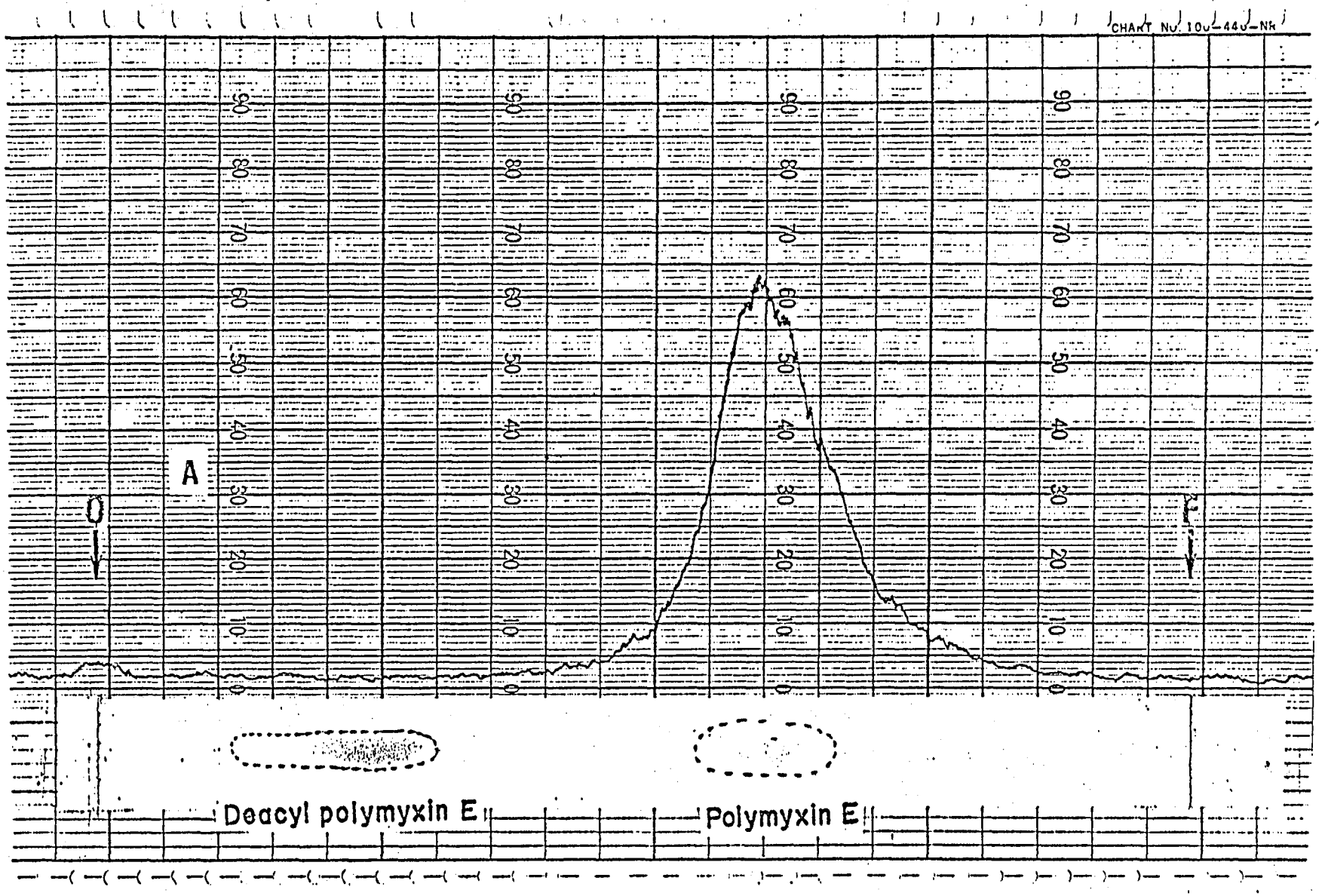
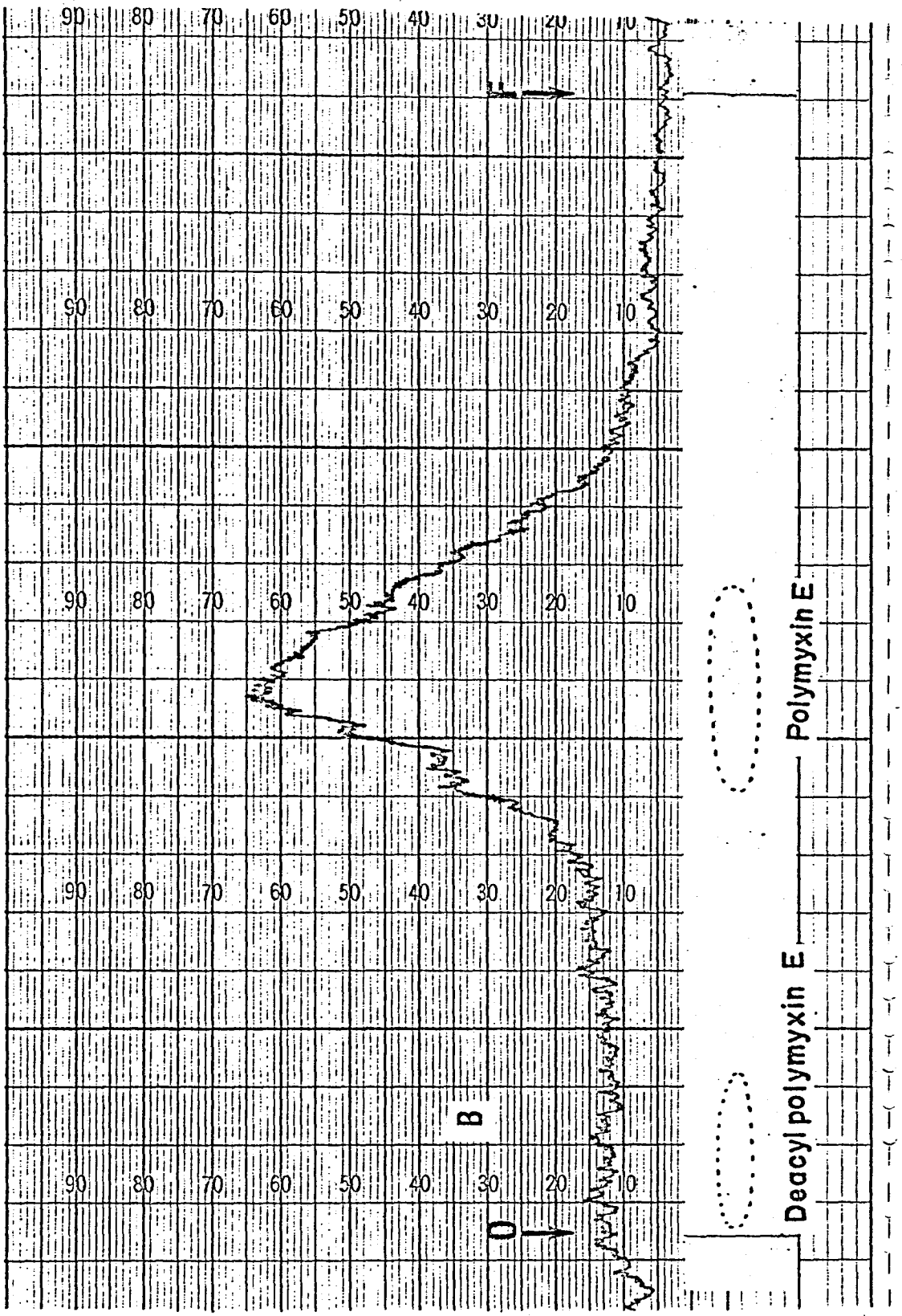


Fig. 2. Paper chromatography of the Effluent from Bio-Gel P-2 Column. Paper chromatography was carried out as described in MATERIALS AND METHODS. (A) Solvent A; (B) Solvent B. The radioacitvity was scanned with a Parckard radiochromatogram scanner Model 7201. The markers, polymyxin E and deacylpolymyxin E, were visualized by ninhydrin spray.


90 80 70 60 50 40 30 20 10
B
O
Deacyl polymyxin E
Polymyxin E

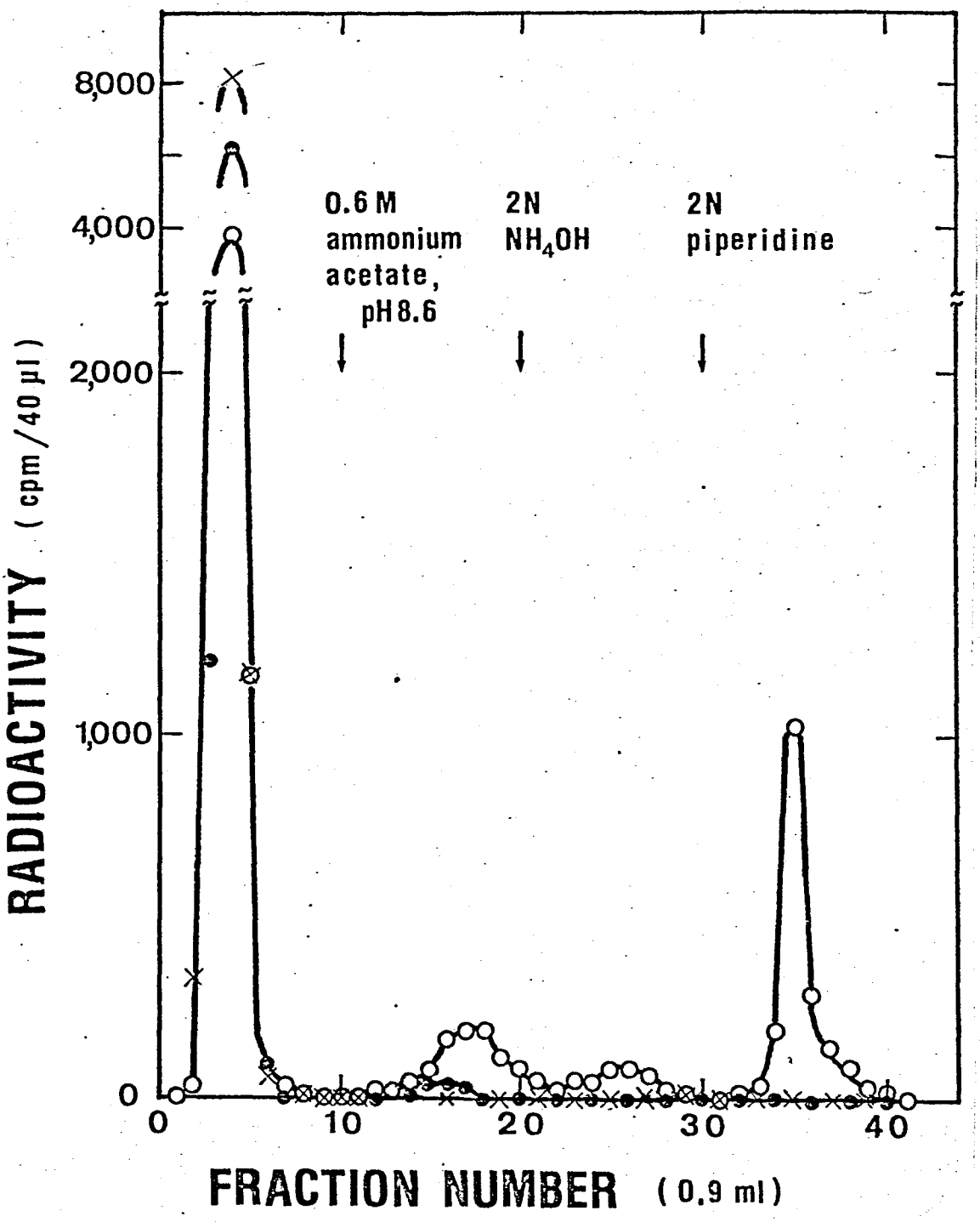


Fig. 3. Dowex 50W-X4 Column Chromatography of the Reaction Products. The supernatant solution of the reaction mixture was obtained as described in MATERIALS AND METHODS and applied to a Dowex 50W-X4 column (NH^4-form, 1 x 2 cm) previously equilibrated with 0.4 M ammonium acetate, pH 5.2. The column was eluted stepwise with 8 ml of the following solution: (1) 0.4 M ammonium acetate, pH 5.2, (2) 0.6 M ammonium acetate, pH 8.6, (3) 2 N NH_4OH, and (4) 2 N piperidine. Forty microliters of each fraction were applied to a Whatman GF/C glass fiber paper, and the radioactivity was determined with a liquid scintillation counter. - o -, complete reaction mixture; - • -, L-DAB, L-threonine and OA-CoA were omitted; - x -, no incubation.

ACKNOWLEDGEMENTS

The author would like to acknowledge the continuing guidance and encouragement of Dr. Kiyoshi Kurahashi.

The author is indebted to Dr. Tomoji Suzuki for drawing his attention to this study.

The author also wish to thank Dr. Yoshitaka Kuriki for his helpful advice.