was much sharper than the descending patterns shown in Fig. 1; in monochloroacetate the reverse obtained. At *p*H 4.2 in μ 0.10 sodium acetate the two patterns were enantiographic.

Diffusion Studies.—The diffusion coefficient was determined at one concentration; 0.4% in 0.15 *M* NaCl at *p*H 3.1. A value of D_{20w} of 1.0×10^{-7} was obtained from the height and area method of calculation. The value calculated using the inflection point method was 0.8×10^{-7} while the method of moments gave $D_{20w} = 3.1 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. The normalized refractive index gradient distance curve did not show skewness but did differ from the ideal curve with respect to the height of the maximum ordinate. This can be taken as evidence of polydispersity.[23] The diffusion results are subject to question, however, for the same reasons as are the sedimentation studies.

Other Studies.—The only criteria of molecular kinetic homogeneity available to us at this time are electrophoresis and turbidity[24] studies. The results of the turbidity measurements made on L3 are consistent with the viewpoint that we are dealing with a single substance.

Sedimentation velocity experiments were run at several ionic strengths at *p*H 3.0. Because the solubility of the material decreased with an increasing concentration of inorganic ions the greatest ionic strength employed was 0.15. Since we were unable to reduce the primary charge effect[25-27] to a negligible amount it was concluded that these experiments did not provide worthwhile information concerning the sedimentation properties of L3.

The intrinsic viscosity (*H*) is indicative of pronounced molecular asymmetry or marked hydration or both. The ultraviolet absorption behavior of L3 is that of a typical protein with a maximum at 278 mμ and a minimum of 250 mμ.

The electron microscope studies were difficult to interpret. Several possible artifacts must be considered. In preparing the sample for examination both drying and shadowing may produce alterations in size and shape. Exposure to high intensity radiation may likewise alter the molecule; L3 has been shown to be unusually sensitive to ultraviolet radiation.[15] Both rods and spheres were observed in the electron micrographs at a magnification of 23,000. The rods were calculated to be approximately 1800 Å. in one dimension and 400 Å. in the other. Such a particle would have a volume about six times that of tobacco mosaic virus obviously inconsistent with the observed molecular weight. If one assumes a rod-shaped molecule and uses the value of $Z = 1.5$ a particle with a dimension of 1400 Å. may be calculated. The diameters of the spheroids were approximately 450 Å. Other particles of various shapes were also observed. These particles appeared to be aggregates of the spheroids. Spheres arranged like beads on a string or in the form of T's, X's and V's were prevalent.

Acknowledgments.—The authors are grateful to Dr. Virgil Koenig for the sedimentation velocity studies as well as for helpful discussions. The electron microscope studies were made with the very helpful assistance of Miss Judith Lindstrom and Dr. R. Q. Blackwell of the School of Dentistry, Northwestern University.

CHICAGO, ILLINOIS

(23) H. Neurath, *Chem. Revs.*, **30**, 374 (1942).
(24) E. L. Hess and D. S. Yasnoff, THIS JOURNAL, **76**, 931 (1954).
(25) T. Svedberg and K. Pedersen, "The Ultracentrifuge," Oxford, Press, New York, N. Y., 1940, p. 23.
(26) L. Varga, *J. Biol. Chem.*, **217**, 651 (1955).
(27) We have estimated from the electrophoretic mobility and the molar frictional constant that at *p*H 3.0 and $\mu = 0.15$ the effective charge of the molecule is still about 6% of the total indicated in reference 19 or approximately 150 protonic charges. A charge of this magnitude would certainly affect the sedimentation behavior of L3. In fact the sedimentation patterns bore a striking resemblance to the pattern obtained by Varga[26] with hyaluronic acid at μ 0.02 where the estimated effective charge was 173.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Amino Acid Sequence of Polymyxin B_1

BY WERNER HAUSMANN

RECEIVED MARCH 16, 1956

Totally substituted DNP-polymyxin B_1 was partially hydrolyzed in acid and the resulting peptide mixture was fractionated by means of multiple dialysis and countercurrent distribution in various systems. Fourteen DNP-peptides were isolated in a state of purity indicated by the criteria of countercurrent distribution, paper chromatography and paper electrophoresis, where the latter technique was applicable. Resubstitution of these peptides with fluorodinitrobenzene and subsequent study by countercurrent distribution and paper chromatography before and after hydrolysis permitted determination of the positions of all the amino acids save one, for which two possibilities still exist. Di-DNP-α,γ-diaminobutyric acid was found to be destroyed to the extent of 60–65% under the hydrolysis conditions. By the same treatment DNP-threonine was 95% converted to dinitroaniline.

Introduction

In an earlier communication[1] it was shown that upon total hydrolysis of polymyxin B_1 (PMB_1) six moles of predominantly L-α,γ-diaminobutyric acid (DAB), two moles of L-threonine (Thr), one mole of L-Leucine (Leu), one mole of D-phenylalanine (Phe) and one mole of 6-methyl-octan-1-oic acid, an isopelargonic acid (Ipel), were liberated. An empirical formula $C_{56}H_{99}O_{14}N_{16}$ for the free base was found and a molecular weight of 1220, assuming the fatty acid residue to be attached to one of the amino groups and the presence of one free carboxyl group in the peptide molecule. The present paper reports experiments designed to determine the amino acid sequence and additional fine

(1) W. Hausmann and L. C. Craig, THIS JOURNAL, **76**, 4892 (1954).

structure of PMB_1. Since pepsin and trypsin were found not to attack the molecule, partial hydrolyses of PMB_1 and dinitrophenyl (DNP)—PMB_1 were carried out in acid.

Experimental

Partial Hydrolysis of Polymyxin B_1.—One gram of PMB_1 hydrochloride was hydrolyzed for 6 hours at 80° in 100 ml. of 6 *N* HCl in a sealed, evacuated tube. The partial hydrolysate was studied by countercurrent distribution (C.C.D.) in the system 2-butanol–1% trichloroacetic acid. After 111 transfers the bulk of the material was found to have travelled slowly as one band (K = 0.18) since it consisted of many strongly basic peptides. There was, however, a second band travelling with a K of 4. The upper phase was evaporated and HCl was added to the remaining lower phase. The trichloroacetic acid was then extracted with ethyl ether. When the aqueous phase was evaporated, colorless needles separated, which gave a single, well defined spot when studied by paper chromatography (P.C.) in the systems pyridine, isoamyl alcohol, water (35:35:30 by vol.)[2] and 2-butanol, 88% formic acid, water (75:15:10 by vol.).[3] The compound was neutral in paper electrophoresis (P.E.). Hydrolysis and P.C. gave spots for Leu and Phe. The compound was definitely identified as the dipeptide phenylalanyl-leucine by substitution with 1-fluoro-2,4-dinitrobenzene (FDNB), hydrolysis and subsequent P.C. This experiment showed the presence of Leu and DNP-Phe.

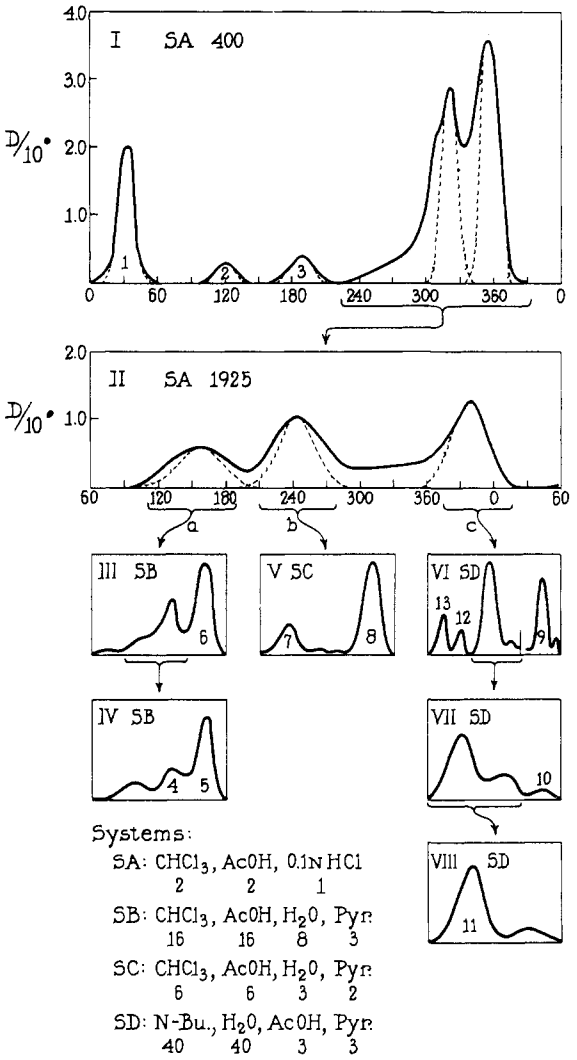

Fig. 1.—Flow sheet of slow dialyzing DNP-peptides from DNP-polymyxin B_1.

Preparation of DNP-Polymyxin B_1.—One gram of PMB_1 was dissolved in 10 ml. of a mixture of acetone, water, triethylamine (6:3:1 by vol.). A great excess of FDNB (2.26 g.) was added and the reaction mixture was kept in the dark at 40° for 15 minutes with occasional shaking. It was then evaporated at 25° and 9 mm. in a rotating evaporator[4] and the residue distributed in the system acetic acid, benzene, water (5:5:1 by vol.). After 215 transfers analysis was carried out spectrophotometrically at 350 mμ. The main band, travelling with a K of 0.762, agreed satisfactorily with the theoretical curve. It was isolated by evaporation to dryness at 25° and 9 mm. and subsequent lyophilization from glacial acetic acid. The figures found by elementary analysis were in agreement with the penta-DNP-polymyxin B_1 (DNP-PMB_1). Tests for free amino nitrogen with nitrous acid were negative.

Anal. Calcd. for $C_{86}H_{109}N_{26}O_{34}$: C, 50.50; H, 5.36; N, 17.77. Found: C, 50.34; H, 5.21; N, 17.59.

The compound was homogeneous by P.C.[2,3] After total hydrolysis for 24 hours at 110° in 12 *N* HCl–acetic acid (1:1 by vol.), P.C. in the same systems showed spots for DAB, Thr, Phe, Leu and γ-DNP-α,γ-diaminobutyric acid (γ-DNP).

Partial Hydrolysis of DNP-Polymyxin B_1.—One gram of DNP-PMB_1 was dissolved in 100 ml. of glacial acetic acid, and the same volume of 12 *N* HCl was subsequently added. Degradation for five days at 37° was chosen on the basis of preliminary experiments. At this point, P.C. showed spots of many yellow DNP-peptides, but also of several unsubstituted peptides and of all the amino acids. The partial hydrolysate was then evaporated in a rotating evaporator at 37° and 9 mm. and lyophilized from glacial acetic acid.

Multiple Dialysis of the Partial Hydrolysate.—The material obtained was dissolved in 24 ml. of a mixture of glacial acetic acid and water (2:1 by vol.) and dialyzed against 240 ml. of the same solvent in two cells, similar to those described by Craig and King.[5] The escape curve was followed through analysis by weight and absorption at 350 mμ. After six hours the solutions inside the cellophane bags contained only the relatively slow dialyzing DNP-peptides and were practically free of colorless peptides and amino acids. The solutions outside the bags were concentrated to 24 ml., put inside two fresh bags and dialyzed as before. By means of three such dialysis procedures it was possible to obtain about half (512 mg.) of the original partial hydrolysate free of colorless, undinitrophenylated fragments, as determined by P.C.

C.C.D. of Slow Dialyzing DNP-Peptides.—The slow dialyzing DNP-peptides were then fractionated extensively by C.C.D. in various systems. Figure 1 summarizes this work in schematic form. The initial step was a 1925 transfer distribution in the system chloroform, acetic acid, 0.1 *N* HCl (2:2:1 by vol.). After the first 400 transfers (pattern I) bands, 1, 2 and 3 were withdrawn and the remainder was recycled. At the end of the run (pattern II) the material was isolated in three cuts, each of which was still a mixture. Cut (a) was redistributed in the system chloroform, acetic acid, water, pyridine (16:16:8:3 by vol.) and gave patterns III at 813 transfers and IV at 2342 transfers. Cut (b) from II was redistributed in the system chloroform, acetic acid, water, pyridine (6:6:3:2 by vol.) and gave pattern V after 1173 transfers. The most polar fraction (c) from II gave patterns VI, VII and VIII after 347, 816 and 1136 transfers, respectively, upon redistribution in *n*-butanol, water, acetic acid, pyridine (40:40:3:3 by vol.). Minimum molecular weights[6] were determined on all the experimental bands which agreed satisfactorily with their calculated curves. They were then tested for purity by P.C. and P.E. where applicable. All those which gave a single spot were totally hydrolyzed under standard conditions, namely, in 12 *N* HCl–CH_3COOH (1:1 by vol.) for 24 hours at 110°. They were then studied by quantitative P.C. in the pyridine and the formic acid systems, using a Photovolt photoelectric densitometer to read the ninhydrin color directly on the

(2) K. Heyns and G. Anders, *Z. physiol. Chem.*, **287**, 1 (1951).
(3) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).
(4) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).
(5) L. C. Craig and T. P. King, THIS JOURNAL, **77**, 6620 (1955).
(6) A. R. Battersby and L. C. Craig, *ibid.*, **73**, 1887 (1951).

paper after development of the spots under standard conditions. In order to detect the N-terminal amino acid, the peptides were substituted further with FDNB, purified by C.C.D. and studied by P.C. before and after total hydrolysis. All the DNP-amino acids which may arise from PMB_1 are well separated on a two-dimensional paper chromatogram using Whatman No. 3MM, run first in 0.2 *M* phthalate buffer of *p*H 6, then in tertiary amyl alcohol saturated with water. In cases where there were indications of destruction of DNP-amino acids during hydrolysis, the hydrolysate was distributed to determine the recovery quantitatively and to investigate the degradation products.

C.C.D. of a Hydrolysate of DNP-Peptide 7.—Figure 2 shows a distribution pattern of a hydrolysate of peptide 7 after further dinitrophenylation. It was not clean cut. The γ-DNP band was fairly sharp but the band of the di-DNP-α,γ-diaminobutyric acid (Di-DNP) was inhomogeneous. The presence of a band in the position of dinitrophenol (DNPOH) aroused suspicion of degradation during hydrolysis.

In a control experiment with synthetically prepared di-DNP-α,γ-diaminobutyric acid treated under the same hydrolysis conditions, the recovery of the starting material was only 40%.

In a similar control experiment with γ-DNP-α,γ-diaminobutyric acid, the recovery was 80%.

C.C.D. of a Hydrolysate of DNP-Peptide 13.—Peptide 13 was further substituted with FDNB and purified by C.C.D. Although both Thr-residues had been dinitrophenylated as indicated by P.C., no DNP-Thr could be found upon C.C.D. of the hydrolysate in the system chloroform, acetic acid, 0.1 *N* HCl (2:2:1 by vol.). DNP-Thr would travel with a *K* of 2 in this system. Instead a band travelling with a partition ratio of 0.77 was present. This solute turned out to be 2,4-dinitroaniline ($DNPNH_2$). In order to determine the stability of DNP-Thr to hydrolysis, a sample of analytically pure DNP-Thr was hydrolyzed under the standard conditions and distributed in the above mentioned chloroform system. Only about 5% of DNP-Thr survived. The rest was transformed with the formation of dinitroaniline.

Four of the peptides were obtained in high enough yield to be checked by elementary analysis.

Compound 1: *Anal.* Calcd. for $C_{19}H_{28}N_4O_7$: C, 53.8; H, 6.7; N, 13.2. Found: C, 53.9; H, 6.4; N, 13.4.

Peptide 3: *Anal.* Calcd. for the hydrochloride $C_{35}H_{43}N_{10}O_{13}Cl$: C, 49.6; H, 5.1; N, 16.5. Found: C, 49.4; H, 5.3; N, 16.8.

Peptide 6: *Anal.* Calcd. for the acetate $C_{18}H_{27}N_5O_9$: C, 47.4; H, 6.0; N, 15.3. Found: C, 47.8; H, 5.8; N, 15.5.

Peptide 8: *Anal.* Calcd. for the diacetate $C_{31}H_{32}N_{18}O_{27}$: C, 48.8; H, 5.5; N, 16.8. Found: C, 48.7; H, 5.3; N, 16.4.

Discussion

In the partial hydrolysis experiments required for structural work with peptides a main difficulty stems from the random splitting of each of the peptide bonds. Enzymatic hydrolysis can be much more selective but unfortunately attempts with this approach were unsuccessful with polymyxin B_1. Pepsin was found to have no effect and trypsin acted very slowly if at all.

Before excluding the possibility of applying one of the several known stepwise degradation procedures to PMB_1 on the basis of its supposed cyclic nature, a careful search for either α-carboxyl or α-amino groups was made. For this purpose the peptide was completely substituted with FDNB and hydrolysed. No α-DNP-amino acid could be found. Attempts to cause diazomethane to react with a possible free carboxyl group of the peptide failed.

Thus direct partial hydrolysis by acid appeared to be the only practical way to gain information about the sequence. A certain difficulty was anticipated in this attempt since six of the ten amino acid residues were strongly basic diamino acids.

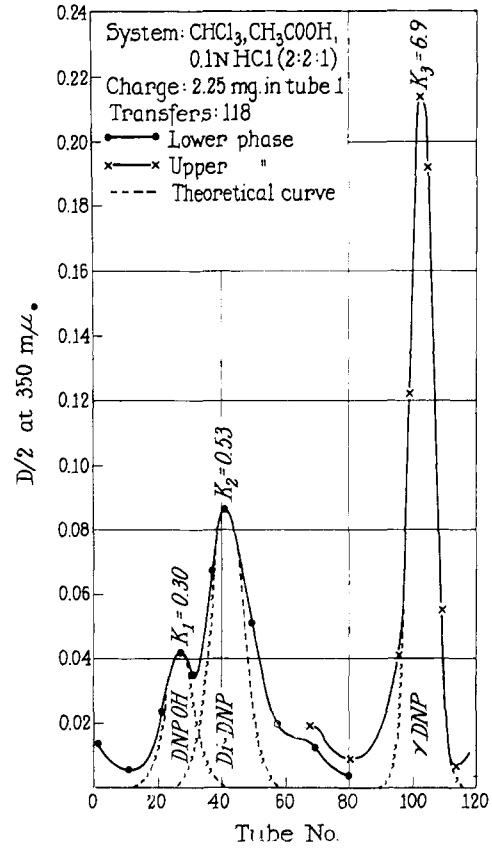

Fig. 2.—Distribution curve of hydrolyzed DNP-peptide 7.

As expected, partial hydrolysis of the peptide gave rise to a complicated mixture of strongly basic peptides with nearly identical partition ratios in the system used for the attempted separation. Even 2000 transfers failed to show much resolution. It is likely that ion exchange chromatography would have been a more promising method of separation in this case.

One approach for improving the separation of strongly basic peptides by C.C.D. is to convert them to DNP derivatives in order to achieve a more favorable polarity balance. Moreover, a second reason for considering this approach was based on the experience of Craig and King[5] in multiple dialysis experiments and in unpublished work. Improved selectivity with this preliminary separation tool was obtained by converting the amino acids and peptides to DNP derivatives.

In the preliminary dialysis experiments with polymyxin B_1 it was found that the intact DNP derivative and DNP partial hydrolysis products when dissolved in acetic acid—water (2:1 by volume), passed through a cellophane membrane at a relatively slow speed as compared to the free amino acids and unsubstituted peptides. It was known from the previous work[5] that poly-basic peptides in acetic acid solution passed through cellophane at an accelerated rate as compared to other solutes of similar size. Thus, as shown in the Experimental part, a few dialysis stages were sufficient to remove essentially all the free amino acids and basic undinitrophenylated peptides from the mixture to be fractionated by C.C.D. and with but little loss of

TABLE I

PEPTIDES FROM PARTIAL HYDROLYSIS OF DNP-POLYMYXIN B_1

No.	Mg.	Mol. wt.	Hydrolysis products	Sequence
1	73.9	380	1 γDNP, 1 Ipel	Ipel → γDNP
		(424)		
2	19.3	1140	3 γDNP, 1 Phe, 1 Leu	γDNP → Phe → Leu → γDNP ↓ γDNP
		(1113)	γDNP, Phe, Leu, DiDNP	
3	44.1	860	2 γDNP, 1 Phe, 1 Leu	Phe → Leu → γDNP → γDNP
		(847)	γDNP, — , Leu, DNP-Phe	
4	5.1	663	2 γDNP, 1 DAB, 1 Thr, 1 Phe, 1 Leu	Thr → γDNP → DAB → γDNP ↓ Leu ← Phe
		(536)	γDNP, — , — , Phe, Leu	
				or
5	11.7	1066	2 γDNP, 1 DAB, 1 Thr, 1 Phe, 1 Leu	Thr → DAB → γDNP → Phe ↓ γDNP ← Leu
		(1072)	γDNP, — , — , Phe, Leu	
6	32.5	500	1 γDNP, 1 Leu	Leu → γDNP
		(458)		
7	16.5	800	γDNP	γDNP → γDNP
		(550)	1 γDNP, 1 DiDNP	
8	56.5	1380	3 γDNP, 1 DAB, 2 Thr, 1 Phe, 1 Leu	Thr → DAB → γDNP → Phe ↑ ↓ Thr → γDNP γDNP ← Leu
		(1500)		
9	13.0	1020	2 γDNP, 1 DAB, 2 Thr, 1 Phe	Thr → DAB → γDNP → Phe ↑
		(1060)	γDNP, DAB, — , Phe, $DNPNH_2$	Thr → γDNP
10	3.8	737	1 γDNP, 1 DAB, 1 Thr, 1 Phe	Thr → DAB → γDNP → Phe
		(692)	γDNP, — , — , Phe	
11	18.0	846	2 γDNP, 1 DAB, 2 Thr	Thr → DAB → γDNP ↑
		(854)	γDNP, DAB, — , $DNPNH_2$	Thr → γDNP
12	4.1	511	1 γDNP, 1 DAB, 1 Thr	Thr → DAB → γDNP
				or
		(600)	γDNP, — , — , $DNPNH_2$	Thr → γDNP → DAB
13	13.0	1010	1 γDNP, 1 DAB, 2 Thr	Thr → DAB ↑
		(707)	γDNP, DAB, — , $DNPNH_2$	Thr → γDNP
(14)	9.5	427	1 Phe, 1 Leu	Phe → Leu
		(444)	— , Leu, DNP-Phe	

yellow material. Even this loss could have been reduced by more dialysis stages. A helpful aspect of having the dinitrophenylated material free of unsubstituted peptides and amino acids was that further fractionation attempts could now be followed easily by optical density measurements at 350 mμ.

In the search for suitable systems for fractionation of the dinitrophenylated peptides by C.C.D. it was found that systems containing various combinations of pyridine and acetic acid were very selective. They permitted a wide variation of the partition ratio due to the different *p*H values of the pyridine acetate buffers.

Figure 1 shows the effectiveness of these systems. All the bands with numbers were found to contain peptides which were homogeneous by C.C.D., and P.C., as well as by P.E. where this was applicable. A total of 13 such peptides was obtained from these experiments. They are listed in Table I according to increasing polarity as indicated by their partition ratios. As expected, this sequence corresponded to an increasing ratio of polar to non-polar groups.

The numbers in column 1 refer to the bands in Fig. 1. In column 2 are given the amounts isolated. The molecular weights are listed in column 3. The calculated values are given in parentheses. For unknown reasons the determined molecular weights of peptides 4, 7, 12 and 13 fell outside the range of ± 10% accuracy claimed for the method. The amino acids indicated by P.C. after hydrolysis both before and after a second treatment with FDNB are listed in column 4. Column 5 contains the sequences derived.

Compound 1 for example gave only one spot for γ-DNP on P.C. after hydrolysis. The partition ratio in the chloroform—acetic acid—0.1 *N* HCl system, the analytical data and the molecular weight indicated the presence of the isopelargonic acid (Ipel) residue and one mole of γ-DNP in the molecule. The structure Ipel →γ-DNP was therefore assigned (column 5). Here, as in all formulas, the point of the arrow is at the amino end of the peptide bond.

Peptide 3 gave spots for γ-DNP, Phe and Leu prior to further DNP-substitution. Two moles of γ-DNP were present. After DNP-substitution and hydrolysis the Phe spot was missing, but DNP-Phe was found instead. Since from experiments described earlier the sequence Phe→Leu was known in PMB_1, the structure in column 5 could be unequivocally assigned.

Peptide 6 contained Leu and 1 mole of γ-DNP.

Since Phe was known to cover the amino end of the Leu, the peptide must have the structure Leu→ γ-DNP.

Peptide 7 was interesting. It gave only γ-DNP upon hydrolysis and it had to be decided whether it was the di- or the tripeptide. Upon further DNP-substitution and hydrolysis, the dipeptide would theoretically give Di-DNP and γ-DNP in a 1:1 ratio, the tripeptide in a 1:2 ratio. When the results from the pattern in Fig. 2 were corrected for the losses by destruction during hydrolysis, as determined by control experiments described earlier, a 1:1 ratio of Di-DNP to γ-DNP was found. The compound therefore was the dipeptide.

Peptide 8 was an octapeptide containing three moles of γ-DNP as indicated by the molecular weight found.

Peptide 13 was basic in P.E. and gave one mole of γ-DNP, one mole of DAB and two moles of Thr upon hydrolysis. After DNP substitution and hydrolysis the γ-DNP and the DAB were still present, but both Thr had disappeared and therefore had to be N-terminal residues. Practically no DNP-Thr could be detected in the hydrolysate, but dinitroaniline ($DNPNH_2$) was found instead. Only the structure given in column 5 was compatible with all the other sequences found.

Using the same reasoning and techniques, structures were assigned to 8 more peptides.

Of the 14 fragments isolated and identified, only 7 key peptides were necessary for structural deductions. They are given in Fig. 3. In it the sequence of every peptide is unequivocally proved directly or in conjunction with the sequences above it. If six of them are put together, the nona peptide at the bottom results. This peptide has a branched chain with a completely covered residue of DAB. All the other sequences found were in agreement with it. The lack of a carboxyl group free or covered with ammonia in intact PMB_1 indicated that a ring was present. The compound Ipel→γ-DNP could only be explained by a tail

No.	Sequence
14	Phe → Leu
6	Leu → γDNP
3	Phe → Leu → γDNP → γDNP
2	γDNP → Phe → Leu → γDNP → γDNP
10	Thr → DAB → γDNP → Phe
9	Thr → DAB(↑γDNP, ↑Thr) → γDNP → Phe
1	Ipel → γDNP

Thr → DAB(↑γDNP, ↑Thr) → γDNP → Phe → Leu → γDNP → γDNP

Ipel → γDNP

Fig. 3.—Seven key peptides.

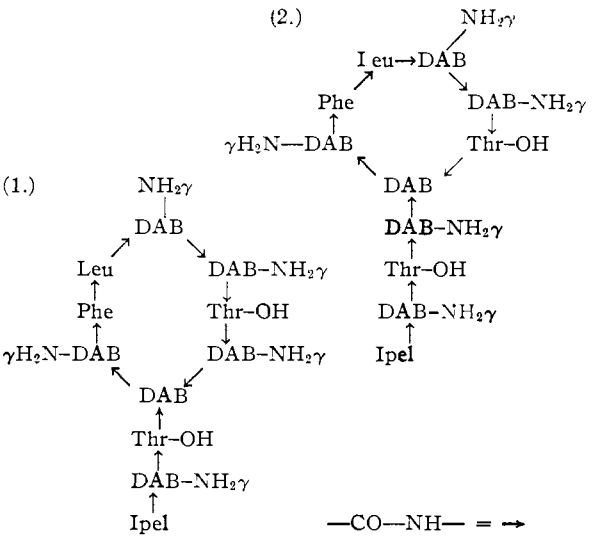

Fig. 4.—Tentative structures of polymyxin B_1.

branching off that ring somewhere. It remained uncertain, however, which Thr-residue the Ipel→ γ-DNP was linked to, depending on which Thr was involved in the ring formation.

Therefore the two structures for PMB_1 shown in Fig. 4 fulfil all the requirements found to date. The one on the left consists of a large ring made up of eight amino acids and a tail ending with the isopelargonic acid. The junction between ring and tail is made by a completely covered residue of DAB. The strong basicity of the compound is evidenced by the presence of five γ-amino groups and the complete lack of acidic functions. The structure on the right is very similar. The only difference is that one DAB-residue is located in the tail instead of the ring. In order to decide between the two, for instance the peptide Ipel→DAB→Thr →DAB→DAB→Phe would be helpful in being compatible with structure 1 only. Unfortunately the peptide bonds involving the amino group of Thr are weak towards mineral acid and not a single Thr-peptide could be found, where this amino acid was not N-terminal. Cleavage by a broad spectrum enzyme such as papain or ficin, is perhaps indicated. Another point in doubt is the manner in which the completely covered DAB-residue is linked, *e.g.*, whether the α- or the γ-amino group is involved in the ring formation.

In conclusion the author wishes to thank Dr. L. C. Craig for many helpful discussions, Dr. H. Jaffe for his assistance in the spectral studies and Miss Gerti Walker for her able technical help. The elementary analyses were carried out by Mr. D. Rigakos.

NEW YORK, N. Y.