790. *The Identities of the Antibiotics Colistin and Polymyxin E.*

By S. Wilkinson and L. A. Lowe.

Polymyxin E and colistin have been shown to be composite, and 6-methylheptanoic acid, in addition to 6-methyloctanoic acid, has been identified in their hydrolysates. Proof of the identities of the two antibiotics is provided by physical and structural determinations.

Six antibiotic peptides, polymyxins A, B, C, and E (discovered in The Wellcome Research Laboratories [1]), polymyxin D,[2] and the more recent polymyxin M,[3] have been isolated from the metabolic products of different strains of the soil isolate *Bacillus polymyxa* [4] (Prazmowski) Migula (*Bacillus aerosporus* Greer).*

The simultaneous announcement in 1947 of the isolation, from related organisms, of "aerosporin" by Ainsworth, Brown, and Brownlee [5] and of "polymyxin" by Benedict and Langlykke [6] and by Stansly, Shepherd, and White [2] raised the inevitable question of chemical identity. A series of co-operative studies, forming the basis of a symposium,[7] showed that the two antibiotics were not identical but closely related chemically and practically indistinguishable biologically. These observations, together with the isolation from other strains of *B. polymyxa* of further peptide antibiotics all having a common pattern of chemical constitution, led to the adoption of the generic name "polymyxin" for this group of antibiotics. The individual members were then characterised by the addition of a suffix to the common name. Thus, "aerosporin" became polymyxin A, and "polymyxin" became polymyxin D.[8] The subsequent rejection of polymyxins A and D because of nephrotoxicity led to the development of the more clinically acceptable polymyxins B and E.[9] In 1956 a strain of *B. polymyxa* isolated from a sample of soil taken in Moscow yielded a further member of the series, polymyxin M.[3]

The polymyxins are cyclic basic peptides, each of molecular weight *ca.* 1200,[10] having neither free α-amino- nor free carboxyl-groups. Their basicities are related to the presence of the uncommon amino-acid αγ-diaminobutyric acid [11] and all contain threonine and a fatty acid, (+)-6-methyloctanoic acid,[12] present as an amide attached to one of the αγ-diaminobutyric acid residues.† Their differences are illustrated in Table 1, which includes circulins A and B, also assigned to this group of antibiotics.[13]

* *B. aerosporus* is not a well-known term and is considered to be a minor synonym of *B. polymyxa*.[4]

† The following abbreviations are used in the text: Ipel = isopelargonic acid = (+)-6-methyloctanoic acid, FDNB = 1-fluoro-2,4-dinitrobenzene, DNP = dinitrophenyl, DNPh = 2,4-dinitrophenol, DNAn = 2,4-dinitroaniline, dab = αγ-diaminobutyric acid, thr = threonine, leu = leucine, phe = phenylalanine.

The abbreviated forms of the amino-acids refer to the L-configuration unless otherwise stated.

[1] Brownlee and Jones, *Biochem. J.*, 1948, **43**, XXV.
[2] Stansly, Shepherd, and White, *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.
[3] Il'inskaya and Rossovskaya, *Antibiotiki*, 1958, 10; Khokhlov and co-workers (see *Chem. Abs.*, 1961, **55**, 5653); *Antibiotiki*, 1960, 3.
[4] Bergey's Manual of Determinative Bacteriology, 6th edn., 1948, William and Wilkins, Baltimore.
[5] Ainsworth, Brown, and Brownlee, *Nature*, 1947, **160**, 263.
[6] Benedict and Langlykke, *J. Bacteriol.*, 1947, **54**, 24.
[7] Report of symposium, Antibiotics derived from *B. polymyxa*, *Ann. New York Acad. Sci.*, 1949, **51**, 853.
[8] Brownlee and Stansly, *Nature*, 1949, **163**, 611.
[9] Bushby, B.P. 646,258 (1950); Wilkinson, B.P. 645,750 (1950); B.P. 658,766 (1951); Diamond, B.P. 742,589 (1955).
[10] Polymyxins A, B, D, and E, Few and Schulman, *Biochem. J.*, 1953, **54**, 171; polymyxin B, Hausmann and Craig, *J. Amer. Chem. Soc.*, 1954, **76**, 4892; polymyxin M, Stepanov, Silaev, and Katrukha, *Biochem. (U.S.S.R.)*, 1961, **25**, 573.
[11] Catch and Jones, *Biochem. J.*, 1948, **42**, LII.
[12] Wilkinson, *Nature*, 1949, **164**, 622.
[13] Sinitsyna and Mamiofe, *Russ. Chem. Rev.*, 1962, **31**, 108.

TABLE 1.

Constitution of polymyxin antibiotics.

Peptide	dab	leu	phe	thr	Ipel	Water-soluble base	Optical rotation		Ref.
Polymyxin A	L and DL	D	—	L	+	+	$[\alpha]_D$	−42° *	14
,, B	L and DL	L	D	L	+	—	$[\alpha]_{5461}$	−106·3 †	15
,, B_1	5L and 1D	1L	1D	2L	1	—	$[\alpha]_D$	−85·1 ‡	16
,, B_2	+	+	+		6-Methyl-heptanoic acid	—	—		16
,, C	+	—	+	+	+	+	—		17
,, D	5L	1D	(1D-ser)	3L	+	+	$[\alpha]_D$	−40 *	18
,, E	6L	1L and 1D	—	2L	+	—	$[\alpha]_{5461}$	−98·7 †	
,, M	6L	1D	—	3L	+	+	$[\alpha]_D$	−48·1, −61·6, −60·6	3, 19, 20
Colistin	5L	1L and 1D	—	1L	+	—	$[\alpha]_D$	−60	21, 22, 23
,,	4	1	—	1L	+	—	—		24
,,	6L	1L and 1D	—	2L	+	—	—		25, 26
Circulin A	6L	1D and 1L-ileu	—	2L	+	—	$[\alpha]_D$	−61·6§	27, 28
,, B	6L	1D and 1L-ileu	—	2L	+	—	—		29
Added in Proof.									
Polymyxin B_1	6L	1L	1D	2L	1				30

The figures in the columns give the numbers of residues of the amino-acid or fatty acid in the antibiotic and, where these have not been established, + indicates the presence of the optically uncharacterised amino-acids and L, D, and DL the presence of the optical form of the amino-acid.

* Hydrochloride in water. † Base in N-HCl. ‡ Hydrochloride in 75% ethanol. § Sulphate in water.

In 1954, Hausmann and Craig,[16] by countercurrent distribution, separated polymyxin B into two components, B_1 and B_2, having the same amino-acids but giving on hydrolysis (+)-6-methyloctanoic acid and an optically inactive octanoic acid, respectively. Following this original work, and with the advent of gas chromatography, all batches of polymyxin B which we have examined have been shown to be composite, and, although there is a tendency for the proportions of the constituents to vary in different batches, polymyxin B_1 is always the major component. The existing methods of manufacture do not achieve any separation or apparently alter the ratio of the antibiotics in any given batch.

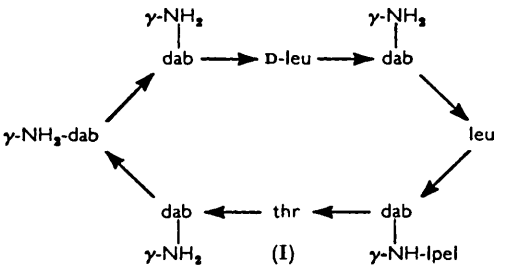
In 1950, Koyama [31-33] extracted an antibiotic from cultures of a soil isolate from Fukuchina Prefecture, Japan. From taxonomic study of the strain, minor differences from *B. polymyxa* were described and a new species named *Bacillus colistinus* (*Aerobacillus colistinus*) was adopted. The antibiotic was called " colistin " and has since been examined extensively in Japan, Italy, and France, also under the name of " colimycin," and has been made available commercially as the sodium sulphomethyl derivatives

[14] Catch, Jones, and Wilkinson, *Ann. New York Acad. Sci.*, 1949, **51**, 917.
[15] Wilkinson, unpublished work.
[16] Hausmann and Craig, *J. Amer. Chem. Soc.*, 1954, **76**, 4892.
[17] Jones, *Ann. New York Acad. Sci.*, 1949, **51**, 909.
[18] Bell, Bone, English, Fellows, Howard, Rogers, Shepherd, and Winterbottom, *Ann. New York Acad. Sci.*, 1949, **51**, 897.
[19] Silaev, Stepanev, Yulikova, Troshko, and Levin, *Zhur. obshchei Khim.*, 1961, **31**, 297.
[20] Khokhlov and Ch'ang-Ch'ing, *Biochem.* (*U.S.S.R.*), 1961, **26**, 267.
[21] Koyama, *Giorn. Ital. Chemioterap.*, 1957, **4**, 279.
[22] Kurihara and K. Suzuki, *Tohoku Yakka Daigaku Kiyŏ*, 1955, **2**, 1.
[23] Suzuki, *Tohoku Yakka Diagaku Kiyŏ*, 1957, **4**, 117 (*Chem. Abs.*, 1958, **52**, 5835).
[24] Dautrevaux and Biserte, *Compt. rend. Soc. Biol.*, 1957, **151**, 1889.
[25] Dautrevaux and Biserte, *Compt. rend. Soc. Biol.*, 1959, **153**, 1346.
[26] Dautrevaux and Biserte, *Bull. Soc. Chim. biol.*, 1961, **43**, 495.
[27] Dowling, Koffler, Reitz, Peterson, and Tetrault, *Science*, 1952, **116**, 147.
[28] Peterson and Reineke, *J. Biol. Chem.*, 1949, **181**, 95.
[29] Koffler, *Science*, 1959, **130**, 1419; *Fed. Proc.*, 1958, **17**, 233.
[30] (*a*) Suzuki, Hayashi, Fujikawa, and Tsakamoto, *J. Biochem.* (*Japan*), 1963, **54**, 555; (*b*) Wilkinson and Lowe, *Nature*, 1264, **202**, 1211; (*c*) Vogler, Studer, Lanz, and Böhni, *Experientia*, 1964, **20**, 365.
[31] Koyama, Jap. P. 1546 (1952) (*Chem. Abs.*, 1953, **47**, 6097).
[32] Koyama, Kurosava, Tsuchiya, and Takakatu, *J. Antibiotics* (*Tokyo*), *Ser. B.*, 1953, **3**, 457.
[33] Ito, Miyamura, Miwayama, Oishi, Igarashi, Hoshino, and S. Muto, *J. Antiobiotics* (*Tokyo*), *Ser. B.*, 1954, **7**, 147.

Colomycin * and Coly-Mycin,* prepared by treating the antibiotic with formaldehyde and sodium hydrogen sulphite.

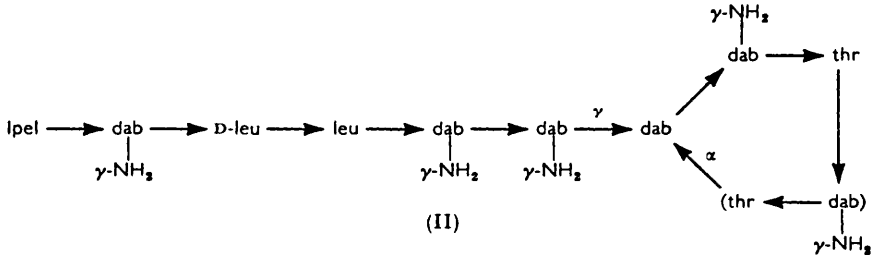
The early chemical investigations showing that colistin was a water-insoluble, cyclic basic peptide giving, on hydrolysis, αγ-diaminobutyric acid, leucine, threonine, and 6-methyloctanoic acid, were sufficient to classify it as a polymyxin although different from polymyxins B and C, which contain phenylalanine, and the water-soluble polymyxins A, D, and M, but closely related to polymyxin E.[34-37]

The number of diverse amino-acid compositions (see Table 1), further analytical figures quoted by Wright and Welch,[38] and the different structures which have been assigned to colistin, are difficult to explain except on the basis of possible batch variations or of variations in preparative procedure. The Japanese workers,[21-23,34,37] from their analyses of the products of partial hydrolysis of colistin, suggested structure (I), a completely cyclic peptide having four free amino-groups.



(I)

However, in a preliminary communication, Dautrevaux and Biserte,[24] working with colistin obtained from Les Laboratories Smit (Turin) and Roger Bellon (Neuilly, Seine), first published the amino-acid composition, thr·leu·$[dab]_4$ but in their more comprehensive Paper [26] assigned the composition $[thr]_2$·leu·D-leu·$[dab]_6$ to the antibiotic. They were unable to confirm either the composition or the structure given by the Japanese workers but found that the amino-acid sequences of their products of partial hydrolysis of colistin and its penta-DNP derivative were indicative of one or other of the two structures (II), in which the alternatives are associated only with the arrangement of the thr and dab in parentheses, and which arise because of the instability, and hence the absence from their hydrolysates, of a peptide such as thr → dab → thr or thr → thr → dab, necessary for complete definition.

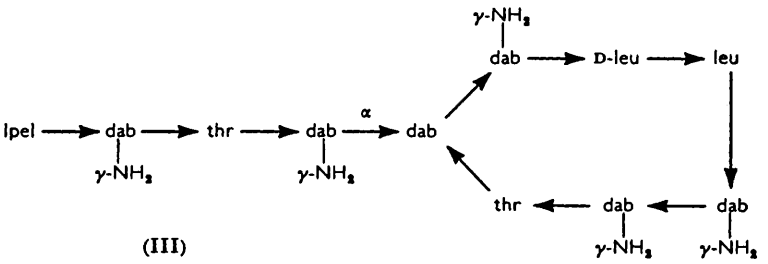


(II)

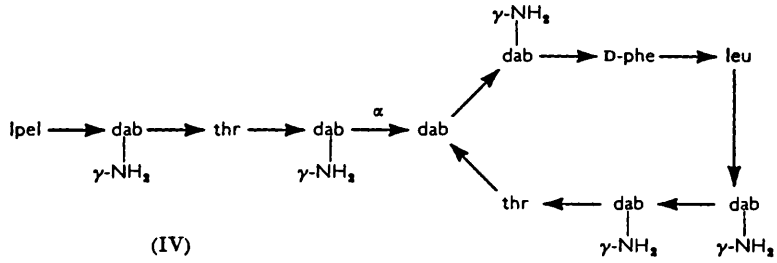
* Registered Trade names, Colomycin (Pharmax, Ltd., Bexley Heath, Kent), Coly-Mycin (Warner-Chilcott, Morris Plains, New Jersey). These names should not be confused with that of the Russian antibiotic, colimycin, which is a member of the neomycin group (Gauze, Kochetkova, Preobrazhenskaya, and Pevzner, *Akad. Med. Nauk. U.S.S.R.*, 1959, 7).

[34] Kurihara and Suzuki, *J. Pharm. Soc. Japan*, 1953, **73**, 414.
[35] Oda, Kinoshita, Yamanaka, and Ueda, *J. Pharm. Soc. Japan*, 1954, **74**, 1243.
[36] Oda and Ueda, *J. Pharm. Soc. Japan*, 1954, **74**, 1246; *Ref. Zhur. Khim.*, 1956, **11**, 253.
[37] Shoji, Hamada, Watanabe, Chiba, Kurozawa, and Koyama, *J. Antibiotics* (*Tokyo*), *Ser. B.*, 1959, **12**, 365 (*Chem. Abs.*, 1960, **54**, 9205).
[38] Wright and Welch, *Antibiot. Ann.*, 1959—1960, 61.

[*Amended in Proof.*] Since the completion of the work here described, Suzuki and his co-workers [39] have shown that colistin is heterogeneous and established that structure (II) for colistin A, the major component, is incorrect and should be replaced by (III).



(III)



(IV)

Degradation of polymyxin E_1 by Suzuki's method is in progress and the results so far parallel those obtained with colistin A, further confirming the identity of the two antibiotics. New information [39–41] establishing the structure of polymyxin B_1 as (IV) reveals a much closer correlation with (III) now suggested for polymyxin E_1.

Results and Discussion

We therefore undertook a comparison of polymyxin E with a sample of colistin obtained from the Warner Lambert Research Institute and also subjected the former to Dautrevaux and Biserte's [26] degradation. When each sample was purified under identical conditions, by demineralisation on an ion-exchange resin, conversion into the base, crystallisation of the derived naphthalene-2-sulphonate, and regeneration of the base, a preliminary examination revealed that in the following properties the two antibiotics were indistinguishable.

Analyses. Elemental analytical figures for the bases, crystalline naphthalene-2-sulphonates, and phosphates corresponded to the formula $C_{53}H_{100}N_{16}O_{13}$ for each of the antibiotics and were distinct from those required for $C_{35}H_{66}N_{13}O_{10}$ first proposed by Dautrevaux and Biserte [24] and for $C_{45}H_{85}N_{13}O_{10}$ given by the Japanese workers.[21-23] The analyses of the salts corresponded to the pentanaphthalanesulphonates and penta-orthophosphates.

Qualitative amino-acid composition. Acid hydrolysis (6N-HCl, 24 hr., 100°) gave only dab, thr, and leu which were identified by paper chromatography [Whatman No. 1 paper, n-butanol–acetic acid–water (120 : 30 : 50)], R_F 0·08, 0·20, and 0·61, respectively.

Quantitative amino-acid composition. Considering the diverse quantitative amino-acid compositions which have been assigned to colistin, considerable effort has been de-

[39] Suzuki, Mayashi, Fujikawa, and Tsukamoto, *J. Biochem.*, 1963, **54**, 555.
[40] Wilkinson and Lowe, *Nature*, 1964, **202**, 1211.
[41] Vogler, Studer, Lanz, and Böhni, *Experientia*, 1964, **20**, 365.

voted to this aspect of the problem and to the determination of the number of free amino-groups. The optical densities at 355 mμ in 1-cm. cells on the Unicam S.P. 600 spectrophotometer of solutions of 34·75 μg./ml. of the dinitrophenyl derivatives of polymyxin E and colistin in 85% aqueous formic acid were 1·02. Applying the figure of 15,000 for the molecular extinction of the dinitrophenyl group, as determined by Battersby and Craig,[42] the calculated equivalent weights of polymyxin E and colistin bases are 238·5.

The acid-free hydrolysates, after heating with 6N-hydrochloric acid for 24 hours at 100° in sealed tubes, were analysed on the Beckmann-Spincel model 120 Automatic

TABLE 2.

Effect of treatment with D-amino-acid oxidase.*

		Before treatment		After treatment *	
		Found (mg.)	No. of residues	Found (mg.)	No. of residues
Colistin (101·5 mg. base)					
Calc. for	mg.				
6 dab	61·58	58·4	5·7	58·7	5·7
2 leu	22·77	22·7	1·99	13·5	1·19
2 thr	20·68	19·7	1·90	19·85	1·92
Polymyxin E (99·21 mg. base)					
Calc. for	mg.				
6 dab	60·12	58·4	5·82	58·1	5·8
2 leu	22·20	22·0	1·99	13·75	1·23
2 thr	20·22	19·7	1·95	20·05	1·98

* D-Amino-acid oxidase (hog kidney) from British Drug Houses, Ltd.

Amino-acid Analyser before and after treatment with D-amino-acid oxidase.[43] The results indicate that both colistin and polymyxin E have the amino-acid composition $[dab]_6$·leu·D-leu·$[thr]_2$.

A check on the configurations of the dab and leu was made by measuring their optical rotations. The free amino-acids were obtained from a hydrolysate of polymyxin by means of IR 4B resin and fractionated on IRC 50 resin buffered at pH 4·7 with N-sodium acetate–acetic acid. From the neutral amino-acids eluted with water, leucine was isolated by precipitation with naphthalene-2-sulphonic acid. The regenerated leu had zero rotation in N-hydrochloric acid, indicating equal proportions of D- and L-leu. Elution of the column with 0·1N-hydrochloric acid gave dab, the monohydrochloride of which had a rotation of $[\alpha]_D^{22}$ +23·4° (*c* 0·8 in 5N-hydrochloric acid). Synthetic L-dab monohydrochloride gives $[\alpha]_D^{22}$ +23·6°, whereas a mixture of 5 L-dab and 1 D-dab would give $[\alpha]_D^{22}$ +15·7°, from which it can be inferred that all the dab present in polymyxin E exists in the L-form.

Infrared spectra. Between 3100 and 650 cm.$^{-1}$ the infrared spectra of colistin and

TABLE 3.

Infrared spectral bands (cm.$^{-1}$) *: E, polymyxin E and colistin; B, polymyxin B.

E		3055		2959	2933	2871	1662	1590	1532	1473	
B	3080	3065	3037	2960	2933	2875	1660		1542	*1474*	*1460*
E	1443	1390	1328	1255	1157	1100 †	970 †	865 †	822		
B	1442	1389	1326	1244	1123		932	*865* †	816 †	*775* †	*742* †

* Inflexions in italics. † Weak and ill-defined bands.

polymyxin E were identical, but differed from that of polymyxin B. The samples, as bases, were prepared as dispersions (0·25% by weight in KCl discs) and their spectra were measured with a Unicam S.P. 100 spectrophotometer with grating accessory.

[42] Battersby and Craig, *J. Amer. Chem. Soc.*, 1952, **74**, 4023.
[43] Meista, *Biochem. Prep.*, 1953, **3**, 66.

The fatty-acid components. Equal weights of fatty acid were obtained from equal weights of polymyxin E and colistin as found by hydrolysis (5N-H_2SO_4, 24 hr., 100°) and titration after steam-distillation, and approximated to 1 mole of acid, $C_9H_{18}O_2$, from one mole of antibiotic, $C_{53}H_{100}N_{16}O_{13}$. Gas chromatography of the hydrolysates of polymyxin B, polymyxin E, and colistin showed that each was heterogeneous, consisting of the same three components, the major one being 6-methyloctanoic acid. The second acid, found in smaller amounts, has now been identified as 6-methylheptanoic acid. The remaining acid (<1%) has been tentatively identified as n-octanoic acid.

These results conflict with the observation made by Dautrevaux and Biserte, who, using paper-chromatographic separation of the hydroxamates of the fatty acids as described by Thompson,[44] were able to detect only 6-methyloctanoic acid in their sample of colistin. Again, the Japanese workers make no reference to the presence of a fatty acid other than 6-methyloctanoic acid. However, at Professor Dautrevaux's request, we examined the sample of colistin on which his structural studies were carried out, and showed that the fatty-acid fraction of the acid hydrolysate gave the usual three peaks in the gas chromatogram, characteristic of the crude fatty acids derived from the polymyxins. Moreover, although the hydroxamate prepared from the crude acid gave only a single spot (R_F 0·90) on the paper chromatogram [Whatman No. 1, n-pentanol–acetic acid–water (40 : 10 : 50)], with the alternative solvent system benzene–formic acid–water (1 : 1 : 1),[44] two spots were obtained, R_F 0·65 and 0·50 (cf. 6-methyloctanoic acid, R_F 0·65).

We have yet to find a sample of a polymyxin or colistin which gives a single fatty acid on hydrolysis, and consider that all samples of those antibiotics consist of mixtures of three components having virtually identical physical properties, which are not separable by normal methods of purification but require techniques such as solvent distribution as described by Hausmann and Craig [16] for the polymyxin B complex. From the hydrolysate of polymyxin B_2 these workers isolated an optically inactive acid, $C_8H_{16}O_2$, which was shown to be different from n-octanoic acid but was not otherwise characterised.

By countercurrent distribution we have now separated both polymyxin B and polymyxin E into their components, B_1 and B_2 and E_1 and E_2.* From polymyxins B_1 and E_1, (+)-6-methyloctanoic acid was isolated. The hydrolysates of polymyxins B_2 and E_2 gave optically inactive fatty acids, $C_8H_{16}O_2$, different from n-octanoic acid and both yielding the same optically inactive amide.

The presence of 6-methyloctanoic acid in polymyxins B_1 and E_1, and the locations of the unknown acids on the gas chromatogram, led, in the absence of a synthetic specimen, to the erroneous conclusions that the C_8 acids might be 5-methylheptanoic acid, dispelled with the synthesis of (+)-5-methylheptanoic acid, which was not racemised under the conditions of hydrolysis and whose amide melted considerably lower than those of the natural acids. The infrared spectrum of the natural amide suggested the presence of an isopropyl function; 6-methylheptanoic acid was therefore synthesised, and its identity with the natural acids confirmed.

Optical rotations. The optical rotations of the antibiotics were virtually identical (Tables 4 and 5). However, polymyxins B_2 and E_2 have somewhat higher optical rotations

TABLE 4.

Optical rotations ($[\alpha]_{5461}^{22}$) of antibiotic bases.

	N-Hydrochloric acid	2% Acetic acid	2M-Acetic acid	Glacial acetic acid
c...	0·9	1·1	0·88	1·5
Polymyxin E	−98·7°	−90·8°	−91·5°	−69·3°
Colistin	−98·5	−89·9	−91·2	−69·5
Polymyxin B	−106·3	−111·7	—	—

* In common with the nomenclature adopted for the components of the polymyxin B complex, we propose to refer to polymyxin E_1 as the antibiotic giving 6-methyloctanoic acid and polymyxin E_2 as giving 6-methylheptanoic acid.

[44] Thompson, *Austral. J. Sci. Res., Ser. B*, 1951, **54**, 180.

TABLE 5.

Optical rotations ($[\alpha]_{5461}^{22}$) of antibiotic base salts.

	c	Polymyxin B	Polymyxin E	Colistin
Sulphate	1·6 (in water)	—	−78·4°	−77·5°
Phosphate	1·0 (in 2% acetic acid)	−82·8	−71·44	−70·76

TABLE 6.

Optical rotations of polymyxins (*c* 1·0 in 2% acetic acid).

		Amino-acid composition				Free NH_2	
Polymyxin	$[\alpha]_{5461}^{22}$	dab	leu	thr	phe	groups	Fatty acid
B_1	−109·6°	6	l	2	lD	5γ	(+)-6-methyloctanoic
B_2	−112·4	6	l	2	lD	5γ	6-methylheptanoic
E_1	−93·3	6	lL + lD	2	—	5γ	(+)-6-methyloctanoic
E_2	−94·5	6	lL + lD	2	—	5γ	6-methylheptanoic

than polymyxins B_1 and E_1, respectively (Table 6). Consequently it is to be expected that small differences in the optical rotations will be observed in different batches of the antibiotics as the proportions of the components vary. Moreover, since we have now found that each common pair of antibiotics has the same amino-acid composition and five free amino-groups associated only with the γ-amino-groups of the dab residues, as shown by hydrolyses of the penta-DNP peptides, it may be anticipated that the rotational differences are associated with the presence of the two different fatty acids.

Reactive Groups. (*a*) A free carboxyl group could not be detected in polymyxin E or in the sample of colistin, using the thiohydantoin method of Dautrevaux and Biserte.[45]

(*b*) The presence of six αγ-diaminobutyric acid residues and the absence of a carboxyl group in polymyxin E and colistin suggest that, provided the fatty-acid residues are present as amides, the antibiotics should have five free amino-groups. When the DNP derivatives of colistin and polymyxin E are hydrolysed [conc. hydrochloric acid–glacial acetic acid (1 : 1 v/v), 100°, 24 hr.] paper-chromatographic and electrophoretic examinations of the hydrolysates reveal the presence of thr, leu, γ-DNP·dab, and dab, but the absence of αγ-DNP·dab, α-DNP·dab, DNP·thr, and DNP·leu. All the free amino-groups in the two antibiotics are therefore identified as the γ-NH_2 groups of the dab residues.

An elegant method of determining the number of amino-groups in polymyxin M and other antibiotics has been devised by Stepanov *et al.*[46] When a polymyxin is treated with insufficient fluorodinitrobenzene (FDNB) for complete reaction, a complex mixture of products of partial substitution is formed. If five amino-groups are present, a range of products from the mono-DNP to the penta-DNP derivatives will be formed. Under suitable electrophoretic conditions these can be separated, there being a stepwise decrease in molecular charge resulting in a corresponding reduction in electrophoretic mobility, and five bands will be detected.* In certain buffers the poor solubilities of the higher DNP derivatives, even though they possess free amino-groups, prevent the migration to the cathode. Thus, in 30% aqueous acetic acid, as was found with polymyxin M and polymyxin B,[46] partially dinitrophenylated polymyxin E and colistin produced only three migrating, yellow bands, together with the unsubstituted antibiotics, which migrated farthest and were located by overspraying with ninhydrin. Stepanov *et al.*[46] demonstrated that, provided minimum migration consistent with an effective separation is achieved (in the neighbourhood of the cathode mobility decreases quite perceptibly) the relative mobilities of the derivatives are related to the number of free amino-groups,

* Account is not taken of isomers differing only in the position of the DNP groups for these will not be separated electrophoretically. Moreover, the free amino-groups in the polymyxins are associated only with the γ-NH_2 groups of the dab residues and not with α-NH_2 groups, which might be of different molecular charge.

[45] Dautrevaux and Biserte, *Compt. rend.*, 1955, **240**, 1153.
[46] Stepanov, Silaev, and Katrukha, *Biochem.* (*U.S.S.R.*), 1962, **26**, 572.

i.e., a monosubstituted derivative will travel $(n - 1)/n$ the distance of the unsubstituted base having n free amino-groups.

A comparison of the calculated and observed relative mobilities indicated that the three migrating derivatives corresponded to the mono-, di-, and tri-DNP antibiotics, and that the tetra- and penta-substituted products did not migrate under the electrophoretic conditions (Table 7).

TABLE 7.

Electrophoretic behaviour of antibiotic dinitrophenyl derivatives in (a) 30% aqueous acetic acid, and (b) 85% formic acid–glacial acetic acid–water (28 : 20 : 52).

	Migration to cathode (cm.)					Relative mobilities					Calc. for	
Antibiotic	Colistin		Polymyxin E		Polymyxin B	Colistin		Polymyxin E		Polymyxin B	5-NH_2	4-NH_2
	a	b	a	b	a	a	b	a	b	a		
Unsubst.	11	8·0	11	8·0	11	1·0	1·0	1·0	1·0	1·0	1·0	1·0
Mono-DNP ...	9·3	6·5	9·3	6·5	9·3	0·84	0·81	0·84	0·81	0·84	0·8	0·75
Di-DNP	7·0	5·0	7·0	5·0	7·0	0·63	0·62	0·63	0·62	0·63	0·60	0·50
Tri-DNP	4·6	3·3	4·6	3·4	4·6	0·42	0·41	0·42	0·42	0·42	0·4	0·25
Tetra-DNP ...	0	1·5	0	1·6	0	0	0·19	0	0·2	0	0·2	0
Penta-DNP...	0	0·0	0	0·0	0	0	0	0	0	0	0 *	0

* In solvent (b); 0·2 in solvent (a).

Electrophoresis in 85% formic acid–glacial acetic acid–water (28 : 20 : 52) as shown by Stepanov *et al.*,[46] gave a better separation, and five distinct yellow bands were obtained, corresponding to the mono-, di-, tri-, tetra-, and penta-DNP derivatives, the last remaining stationary (Table 7). The unsubstituted antibiotic was again located by spraying with ninhydrin.

The results can be checked by the additional procedure devised by Stepanov *et al.*[47] which does not depend on the complete separation of the components of the partial dinitrophenylation. Each of the migrated bands is converted *in situ* into the completely substituted derivatives by reaction with an excess of FDNB in an atmosphere of triethylamine. After the removal of the secondary products of the reaction, the bands are eluted and their optical densities at 355 mμ compared with equivalent amounts of the respective bands prior to treatment with FDNB. Thus, on converting the mono- and di-DNP derivatives into the fully substituted compounds the expected changes in optical density were observed (Table 8).

TABLE 8.

Changes in optical density at 355 mμ (1 cm. cell) on treatment with FDNB.

	Before treatment	After treatment	Ratio
Colistin mono-DNP.....	0·025	0·129	1 : 5·15
Colistin di-DNP	0·041	0·100	2 : 4·98
Polymyxin E mono-DNP	0·028	0·144	1 : 5·14
Polymyxin E di-DNP.....	0·041	0·106	2 : 5·17

The results show that both polymyxin E and colistin have five free amino-groups. In contrast to the behaviour of the DNP derivatives on electrophoresis in 30% aqueous acetic acid, the mixtures of acetyl derivatives of polymyxins B and E and colistin, prepared by treating the bases in glacial acetic acid with insufficient acetic anhydride for complete acetylation, gave five well-defined bands after spraying with ninhydrin, but somewhat displaced, particularly with greater distances of migration (Table 9).

A sample of " Colimycin methane sulphonate " from Kayaku Antibiotics Research Co., Ltd., Tokyo, Japan,* was converted into the free colistin, partially acetylated, and compared with partially acetylated polymyxin E, also regenerated from its sodium sulphomethyl derivative. Five ninhydrin-active bands were found in each case (Table 10).

* " Painless " Trade Mark Colistin.

[47] Stepanov, Silaev, and Katrukha, *Biochem.* (*U.S.S.R.*), 1961, **25**, 573.

TABLE 9.

Electrophoretic behaviour of antibiotic acetyl derivatives.

	Migration to cathode (cm.)			Relative mobilities		
Antibiotic	Polymyxin E	Colistin	Polymyxin B	Polymyxin E	Colistin	Polymyxin B
Unsubst.	8·5	8·5	9·5	1·0	1·0	1·0
Band 1.....	7·45	7·4	8·6	0·87	0·87	0·90
Band 2.....	6·1	6·15	7·0	0·72	0·72	0·74
Band 3.....	4·35	4·3	5·1	0·51	0·51	0·54
Band 4.....	2·3	2·25	2·9	0·27	0·30	0·30

TABLE 10.

Comparison of electrophoretic behaviour of partially acetylated colistin and polymyxin E.

	Migration to cathode (cm.)		Relative mobilities	
Antibiotic	Colistin	Polymyxin E	Colistin	Polymyxin E
Unsubst.	5·4	5·4	1·0	1·0
Band 1.....	4·5	4·5	0·83	0·83
Band 2.....	3·6	3·6	0·66	0·66
Band 3.....	2·5	2·5	0·46	0·46
Band 4.....	1·2	1·2	0·22	0·22

There appears to be little question that the Japanese colistin possesses five free amino-groups and not four as reported.[21-23,33,36]

As a matter of interest, in the plate diffusion assay using *Brucella bronchiseptica* the monoacetyl polymyxins B and E were found to have antibiotic activity but of a lower order than the parents. The more highly substituted derivatives were biologically inactive.

Inactivation with acetic anhydride. When polymyxins B and E were treated with acetic anhydride in glacial acetic acid there was an irreversible loss of antibiotic activity and an increase in optical rotation. Within the limits of error the rates of increase of optical rotation of polymyxin E and colistin were identical but different from that of polymyxin B (Table 11).

TABLE 11.

Change of optical rotation, $[\alpha]_{5461}^{22}$, on treatment with acetic anhydride.

Time (min.)	Polymyxin B	Colistin	Polymyxin E
0	−90° *	−71·5° *	−71·5° *
5	−93·63	−77·69	−77·52
15	−99·31	−85·19	−85·55
25	−101·2	−88·9	−89·10
35	—	−90·78	−90·88
45	—	−91·73	−91·77
55	−102·0	−92·64	−92·66
70	−104·0	−93·61	−93·56
85	−105·0	−94·54	−94·45
100	−105·0	−94·54	−94·45
115	−105·0	−94·54	−94·45
Total increase	−15	−23·04	−22·95

* By extrapolation.

Rates of hydrolysis. The changes of the optical rotations of solutions of polymyxin E and colistin bases in 6N-hydrochloric acid at 22° were followed over a period of 4 days (Table 12).

TABLE 12.

Change of optical rotation, $[\alpha]_{5461}^{22}$ (*c* 0·55), on treatment with 6N-hydrochloric acid.

Time (hr.) ...	0	0·5	1	3·5	6·5	10·5	22·5	26	45	98
Polymyxin E	—	−91·18°	−90·35°	−84·16°	−77·94°	−70·86°	−57·57°	−54·03°	−41·64°	−26·57°
Colistin	—	—	−90·35	−84·16	—	−70·86	−57·57	−53·14	−41·64	−25·68

Electrophoretic and paper-chromatographic behaviour. Electrophoresis of polymyxin E and colistin [Whatman 3MM paper, 360v, 30% aqueous acetic acid and 85% formic acid–acetic acid–water (28 : 20 : 52)] showed that they had the same mobilities (see Table 7).

Differences between the R_F values of polymyxin E and colistin could not be detected provided that, in the various chromatographic procedures, equal amounts were applied as equal-sized spots in correspondingly equivalent positions, the samples were of equal purity and free from inorganic salts, the bases were used in preference to the sulphates or hydrochlorides, and the papers were equilibrated in the solvent vapour prior to development. In particular, the application of different concentrations of the antibiotics affected the R_F values considerably. Even under ideal conditions, the paper-chromatographic identification of peptides of this type, in which somewhat elongated rather than compact spots are obtained, is not in itself entirely satisfactory. The elongations can be somewhat reduced by addition of salts to the eluants.

The R_F values given in Table 13 refer only to those chromatograms in which the solvent travelled limited distances down the paper, but in many runs the times of development were extended and the solvents were allowed to run off the paper. Thus, on Whatman No. 1 paper polymyxin E and colistin both travelled 35·2 cm. in solvent 3 and 31·1 cm. in solvent 2 in 24 hours.

TABLE 13.

Paper-chromatographic data.

Solvent *	Whatman paper no.	Polymyxin A	Polymyxin B	Polymyxin E	Colistin
1	1	0·39	0·54	0·51	0·51
1	2	0·29	0·42	0·40	0·40
1	4	—	0·52	0·51	0·51
1	20	0·15	0·31	0·30	0·30
2a †	1	—	0·50 ‡	0·46 ‡	0·46
2a	4	—	0·46	0·45	0·45
2a	20	—	—	0·20	0·20
2b	1	0·92	0·86	0·90	0·90
3 §	1	0·58	0·73	0·77	0·77
3	4	—	0·73	0·72	0·72
3	20	—	—	0·64	0·64
4	1	—	0·42	0·42	0·42
5	1	—	0·37	0·37	0·37
5	4	—	0·46	0·46	0·46
6	1	—	0·24	0·24	0·24
5	1	—	0·78	0·78	0·78

* 1, n-butanol–acetic acid–water (120 : 30 : 50); 2, n-butanol–acetic acid–water (4 : 1 : 5), (a) upper phase, (b) lower phase; 3, n-butanol–pyridine–acetic acid–water (30 : 20 : 6 : 24);[41] 4, n-butanol–acetic acid–1% aqueous sodium chloride solution (120 : 30 : 50); 5, n-butanol–acetic acid–5% aqueous sodium chloride solution (120 : 30 : 50); 6, n-butanol–acetic acid–water (100 : 10 : 100); 7, n-butanol–pyridine–acetic acid–1% aqueous sodium chloride solution (30 : 20 : 6 : 24). † Dautrevaux and Biserte[24] give polymyxin B, 0·24; polymyxin E, 0·39; colistin, 0·35; § cf. Vogler,[41] Schleicher and Schüll (paper 2043) give polymyxin B_1, 0·57. ‡ Very small, barely detectable differences were observed between the individual pairs of polymyxins: B_2, 0·484; B_2, 0·486; and E_1, 0·464, E_2, 0·465.

At this stage it was apparent that the chemical and physical tests which had been applied had not shown any significant differences between polymyxin E and colistin, even taking into consideration the heterogeneous nature of the antibiotics. Hugo and Stretton[48] also reported on the close similarity between polymyxin E and colistin based on biochemical data, infrared spectra, measurements of surface tension, paper chromatography, and analytical results. It seemed probable that only a comparison of the structures of the two antibiotics would establish identity beyond question, for it was possible that minor structural differences would not necessarily be reflected in large variations in the physical constants. Vogler *et al.*, for example, have shown that the infrared spectra and R_F values of the synthetic 7α, 7γ, 8α, and 8γ-variants of polymyxin B_1 are identical. Differences are reflected in the optical rotations $[\alpha]_D^{25}$: 7α −65·3°, 7γ −60·0°, 8α −57·7°, 8γ −57·5°; polymyxin B_1 −85·1° (in 75% aqueous ethanol).

A preliminary examination by electrophoresis and paper chromatography of partial hydrolysates of polymyxin E and colistin and their penta-DNP derivatives showed such

[48] Hugo and Stretton, *J. Pharm. Pharmacol.*, 1963, **15**, 489.

identities as to warrant pursuing the final structural proof. Thus, partial hydrolysates (conc. HCl, 37°, 6 hr.) gave identical paper chromatograms (Whatman No. 1 paper with solvent 1) with bands having R_F 1·0, 0·84, 0·74, 0·62, 0·50, 0·41, 0·39, 0·30, 0·18, 0·15, 0·098, 0·08, and 0·012 (compared with the reference R_F 1·0 for γ-Ipel·dab) and electrophoretograms (Whatman 3MM paper for 3 hours at 350v in pyridine buffer, pH 3·9) with bands migrating 11·4, 9·6, 8·5, 7·8, 6·6, 5·2, 4·5, 1·3, and 0·06 cm. towards the cathode. These chromatograms and electrophoretograms do not differ extensively from those obtained with a comparable hydrolysate of polymyxin B. Thus, for example, the electrophoretograms differ only in that the bands migrating to 8·5 and 4·5 cm. are absent from the polymyxin B hydrolysate.

Dautrevaux and Biserte,[26] as mentioned previously, were able to identify only 6-methyloctanoic acid in the hydrolysate of colistin. Although we had a reasonable basis for doubting this observation and the inference that these investigators were working with a single entity, we did not have an opportunity to check their observations with this particular batch of colistin until our degradative work had been completed. Therefore, wishing to follow as closely as possible the methods employed by these workers in their determination of the structure of colistin and, moreover, to work with a homogeneous preparation, our sample of polymyxin E was subjected to countercurrent distribution by the procedure described by Hausmann and Craig [16] in their separation of polymyxins B_1 and B_2, and the two comparable polymyxins E_1 and E_2 were isolated. Attention should be drawn to an artefact which arose during the countercurrent distributions of both polymyxins B and E. The periods of manipulation were obviously long and in each case an additional fraction (~1%) was isolated which was not present in the original antibiotic and which we consider to arise as a result of partial hydrolysis in the acidic solvent. Electrophoretically these behave more basically than either polymyxin B_1 or B_2 and polymyxin E_1 or E_2 and their relative mobilities suggest that each possesses an additional free amino-group. Only the fraction obtained from polymyxin B has been examined biologically and has the same order of activity (plate diffusion method) as polymyxin B_1 but as yet no degradative work has been carried out on the material.

Countercurrent distribution of polymyxin E. Polymyxin E base (batch P61/4, 8588 u./mg., 15 g.) was suspended in water (75 ml.), brought into solution by addition of N-hydrochloric acid (to pH 2·9), and freeze-dried. The hydrochloride (10 g.) was dissolved in 300 ml. of the aqueous phase of the mixture n-butanol–0·1N-hydrochloric acid and transferred to the first 15 tubes of a countercurrent distribution apparatus * (240 tubes, 20 ml. upper and lower phases) and subjected to 2056 transfers using the recycling technique. The two phases in each fifth tube was analysed, after suitable dilution, by spectrophotometric measurements at 570 mμ of the colour developed with ninhydrin, according to the procedure described by Yemm and Cocking.[49] The main fraction, polymyxin E_1, was located in tubes 180—224 (peak tube at number 204, $K = 0{\cdot}206$) and polymyxin E_2 in tubes 110—140. Polymyxin E_1 was isolated by removal of the solvent under reduced pressure, dissolution of the residue in water, and basification at 0° with ammonia (*d* 0·88). The base (6 g.) had an activity of 9103 u./mg. and the analysis by gas chromatography of the fatty acid obtained by hydrolysis (5N-H_2SO_4, 24 hr., 100°), showed that the purity was *ca.* 98%.

Analyses of the DNP-peptides from partial hydrolysis of DNP-polymyxin E_1. DNP-Polymyxin E_1 was subjected to partial hydrolysis [glacial acetic acid–concentrated hydrochloric acid (1 : 1, v/v), 37°, 6 days] and the residue after evaporation *in vacuo* separated into ethyl acetate-soluble and -insoluble fractions. The acetate-soluble fraction was further purified, to remove basic constituents the entrained during extraction, by passage through IR-120 H resin, as described by Dautrevaux and Biserte,[40] and then subjected to

* Supplied by J. W. Towers & Co., Ltd., Victoria House, Widnes, Lancs.

[49] Yemm and Cocking, *Analyst*, 1955, **80**, 209.

countercurrent distribution [2·4 g. hydrolysate, 20 ml. upper and lower phases, solvent system acetic acid–chloroform–0·1N-hydrochloric acid (2 : 2 : 1)]. After 234 transfers, both phases in every fifth tube were assayed by measurement of the optical density at 355 mμ after suitable dilution with ethanol, when the distribution pattern summarised in Table 14 was obtained, corresponding to five distinct fractions. Each of the fractions was tested for purity by electrophoresis (Whatman 3MM, 30% aqueous acetic acid, 350v) and paper chromatography (Whatman No. 1, 1·5M-phosphate buffer, pH 6·0) and then analysed as follows:

(*a*) After hydrolysis (6N-HCl, 100°, 24 hr.), the hydrolysates were extracted with ether and the ethereal extracts were tested for the presence of 6-methyloctanoic acid by paper chromatography of the hydroxamate, as described by Thompson.[44]

(*b*) With the exception of fraction 1, shown to be dinitroaniline, paper chromatography of the above hydrolysates revealed in each only γ-DNP·dab and leu, with traces of dinitrophenol which were readily removed by sublimation *in vacuo*. It was found most convenient to separate these two components by electrophoresis (Whatman 3MM, 30% aqueous acetic acid, 360v) and the molar ratios were then estimated. The γ-DNP·dab was eluted with 80% aqueous ethanol containing 1% of concentrated hydrochloric acid, and the optical density of the eluate was determined at 355 mμ. A correction of +11% was applied during the estimation, determined by carrying out a blank hydrolysis using γ-DNP·dab. The leucine was determined by the colorimetric method with ninhydrin.[49]

TABLE 14.

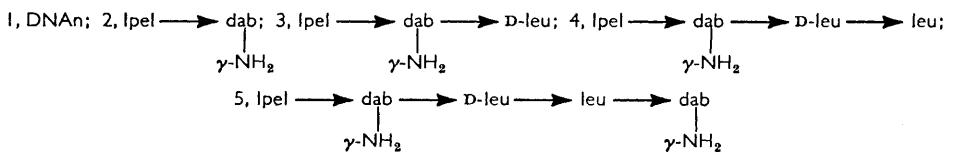
Composition of the ethyl acetate-soluble fraction of the partial hydrolysate of DNP-polymyxin E_1.

			Hydrolysate			After treatment with D-amino-acid oxidase	After treatment with FDNB
Fraction	Tubes	*K*	Ipel	γ-DNP·dab	leu	leu	DNP-leu
1	4—17	0·029	—	—	—	—	—
2	20—50	0·13	+	1	—	—	—
3	55—85	0·46	+	1	1	—	—
4	140—156	1·51	+	1	2	1	—
5	170—185	3·1	+	2	2	1	—

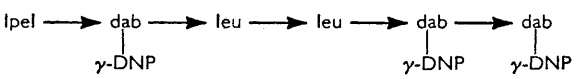
(*c*) The molar ratios of γ-DNP·dab and leu were again estimated after treatment of the leu *in situ* with D-amino-acid oxidase.

(*d*) Each fraction was treated with an excess of FDNB and hydrolysed [conc. HCl–glacial acetic acid (1 : 1 v/v), 100°, 24 hr.]. No DNP·leu was detected in the hydrolysates, paper chromatography revealing only γ-DNP·dab and leu.

From the results in Table 14 the sequences of the amino-acids in the fractions are therefore:



Biserte and Dautrevaux [40] found that, under similar conditions of hydrolysis, DNP-colistin gave the same fragments. In addition, however, from fraction 2, by paper chromatography, they isolated and characterised the DNP-peptide,



In our experiments we also found that fraction 2 gave two components on the paper chromatogram (Whatman No. 1, 1·5M-phosphate buffer, pH 6·0) but that the second component was present in an amount insufficient for satisfactory analysis.

In contrast to the ethyl acetate-soluble fraction, which gave only two bands on electrophoresis (Whatman 3MM, 30% aqueous acetic acid, 5 hr., 360v), 12 migrating bands were found in the acetate-insoluble fraction, and the two electrophoretograms were identical with those obtained from similar fractions of hydrolysed DNP-colistin (Table 15).

TABLE 15.

Electrophoresis (migration in cm.*) of partial hydrolysates; (a) ethyl acetate-soluble and (b) ethyl acetate-insoluble fraction.

DNP-Colistin											
a:	+0·2	0·0	−0·35 (trace)								
b:	+0·3	−2·5	−3·6	−4·0 †	−4·5	−5·4	−6·0	−6·5 ‡	−6·8	−7·3 †	−8·3	−9·6 ‡
DNP-Polymyxin E											
a:	+0·2	0·0	−0·35 (trace)								
b:	+0·3	−2·4	−3·5	−4·0 †	−4·4	−5·4	−6·0	−6·5 ‡	−6·8	−7·2 ‡	−8·3	−9·6 ‡

* +, migration to anode; −, migration to cathode. † Yellow bands becoming blue-green on spraying with ninhydrin. ‡ Colourless bands located by overspraying with ninhydrin.

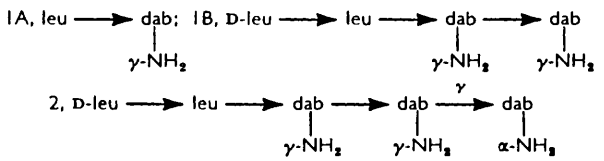
The ethyl acetate-insoluble fraction of the DNP-polymyxin E hydrolysate was also subjected to countercurrent distribution under the same conditions used for the fractionation of the acetate-soluble portion. After 234 transfers, two well-defined peaks were located between tubes 90—115 and 140—170, but there was also considerable trailing and concentration of material in the final 30 tubes. Fractionation of the contents of these final tubes on a cellulose column in n-butanol–acetic acid–water (120 : 30 : 50) yielded γ-DNP·dab, but the remaining fractions were not homogeneous. Tubes 90—115 gave two bands of approximately equal concentration and tubes 140—170 a trace of a second band, in addition to the main one, on electrophoresis. The bands were eluted and analysed as described previously (Table 16).

TABLE 16.

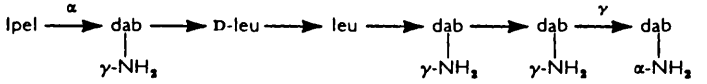
Composition of the ethyl acetate-insoluble fraction of the partial hydrolysate of polymyxin E_1.

		Hydrolysate				After treatment with D-amino-acid oxidase	After treatment with FDNB			
Fraction	Tube	Ipel	dab	leu	γ-DNP·dab	leu	α-DNP·dab	γ-DNP·dab	leu	DNP·leu
1A	90—115	—	—	1	1	1	—	1	—	1
1B		—	—	2	2	1	—	2	1	1
2	140—170	—	1	2	2	1	1	2	1	1

The results in Table 16 indicate that the sequences of the amino-acids in the fractions are:



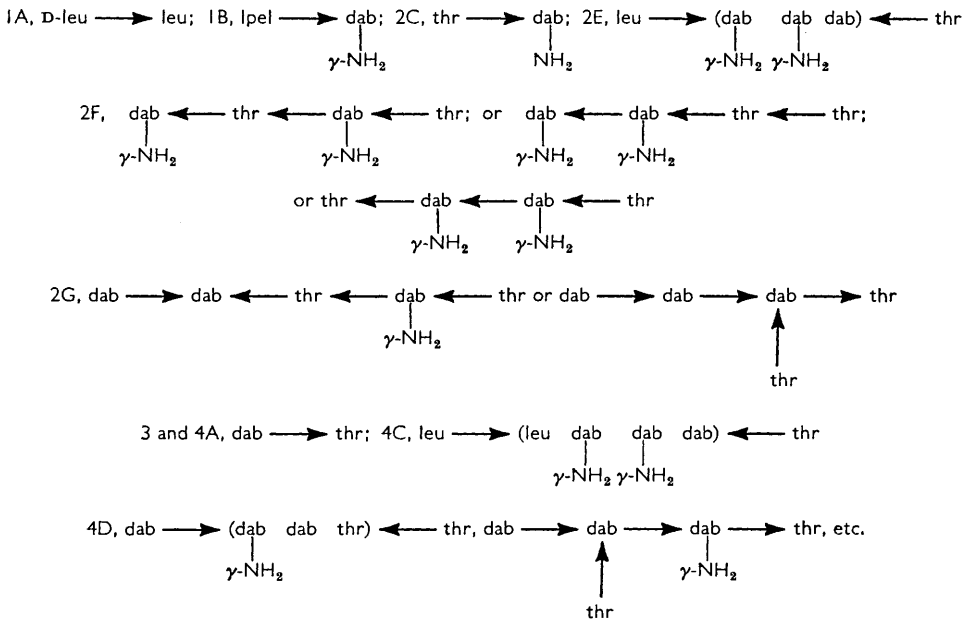
The combined analyses of the partial hydrolysis products of DNP-polymyxin E therefore give the partial structure:



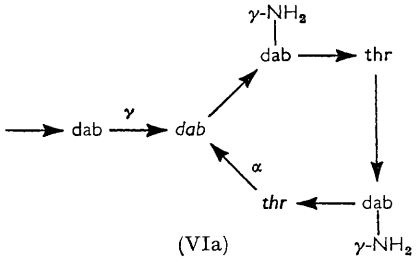
(V)

Analyses of the Peptides from Partial Hydrolysis of Polymyxin E.—Polymyxin E_1 was subjected to partial hydrolysis (conc. HCl, 37°, 6 days). Under these conditions both the hydrolysates of polymyxin E and colistin gave identical chromatograms [Whatman No. 1, n-butanol–acetic acid–water (120 : 30 : 50)] with bands having R_F 1·00, 0·82, 0·67, 0·42, 0·39, 0·34, 0·24, 0·15, 0·12, 0·077, and 0·031 (R_F 1·0 for γ-Ipel·dab) and electrophoretograms [Whatman 3MM, 360v, 4½ hr., acetic acid–85% formic acid–water (20 : 28 : 52)] with bands migrating 12·5, 11·4, 10·7, 10·4, 9·7, 8·0, 7·4, and 5·0 cm. towards the cathode. The acid-free hydrolysate was fractionated on columns of Dowex 50 × 4 resin (150 × 1·2 cm.), prepared according to the procedure of Biserte and Dautrevaux,[40] with gradient elution using the two buffer solutions, ammonium formate (0·05M, pH 2·3) and ammonium formate–ammonium acetate (0·22M, pH 9·26). Two columns, one maintained at 25° and the other at 50°, were used and the fractions from each of the columns were analysed separately. Fractions (5 ml.) of the eluates were examined by paper chromatography, combined where appropriate, and the individual components were separated by paper chromatography and electrophoresis, and analysed as described previously (Table 17). Hausmann [39] recorded the following recoveries on hydrolysis [12N-HCl–glacial acetic acid (1 : 1 v/v) 110°, 24 hr.] of the DNP-amino-acids: αγ-DNP·dab (60—65%), γ-DNP·dab (80%), and DNP·thr (5%, 95% conversion to DNAn). In our experience, carrying out the hydrolysis in nitrogen in sealed tubes gave somewhat higher recoveries, αγ-DNP·dab 70—75%), γ-DNP·dab (90%), and DNP·thr (25%).

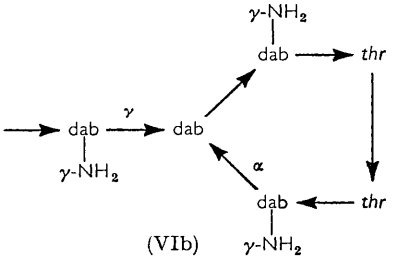
From the analyses in Table 17 the sequences of the amino-acids in the fractions can be interpreted as follows:



From the combined analyses it is possible to assign one or other of the two partial structures (VIa) and (VIb) to polymyxin E. Differentiation would depend on the isolation of the free peptides thr → dab or thr → thr (italicised in the formulæ) from the products of hydrolysis of DNP-polymyxin E. Like Dautrevaux and Biserte,[26] we have been unable to detect either of these peptides, and although the hydrolysates contain traces of unsubstituted derivatives (Table 15), these appear to correspond to the free amino-acids thr and dab. In the opinion of the French workers, the two peptides are too



(VIa)



(VIb)

unstable to resist hydrolysis under the conditions employed. We therefore conclude that both polymyxin E and colistin have one or other of the structures given in formula (II), as derived by combining structure (V) and (VI), and, in the absence of any evidence to the contrary, consider that polymyxin E and colistin are identical.

TABLE 17.

Composition of the partial hydrolysate of polymyxin E_1.

pH	Tubes *	Fraction	R_F †	Migration ‡	Hydrolysate	After treatment with FDNB
5·18	40—129C	1A	0·86	3·2	2 leu §	DNP·leu + leu
	20—100H	1B ¶	0·86	5·3	Ipel + dab	Ipel + γ-DNP·dab
5·18	130—140C	2A		5·4 ‖	dab + leu + thr	
	130—140C	2B		6·2 ‖	dab + leu + thr	
	120—200H	2C	0·20	7·5	1 dab + 1 thr	DNP·thr + γ-DNP·dab
		2D		8·4 ‖	dab + leu + thr	
		2E	0·23	10·3	3 dab + 1 leu + 1 thr	DNP·thr + DNP·leu + γ-DNP·dab + dab
		2F	0·23	11·5	2 dab + 2 thr	γ-DNP·dab + DNP·thr + thr
		2G	0·10	14 **	3 dab + 2 thr	dab + thr + γ-DNP·dab + DNP·thr + αγ-$[DNP]_2$·dab
7·7—8·2	145—155C	3	0·10	5	1 dab + 1 thr	thr + αγ-$[DNP]_2$·dab
8·2	204—220H	4A	0·10	5	1 dab + 1 thr	thr + αγ-$[DNP]_2$·dab
		4B	0·20	5·9 ‖	dab	
		4C	0·20	7	3 dab + 2 leu + 1 thr	DNP·leu + leu + DNP·thr + dab + γ-DNP·dab
		4D	0·13	9·7	3 dab + 2 thr	dab + thr + DNP·thr + γ-DNP·dab + αγ-$[DNP]_2$·dab

* C refers to eluate from column run at 25°, H to column run at 50°. † Whatman No. 1, n-butanol–acetic acid–water (120 : 30 : 50). ‡ Whatman 3MM, 30% aqueous acetic acid, 360v, migration (cm.) to cathode after 6 hr. § Gave 1 leu after treatment with L-amino-acid oxidase (*Crotalus adamanteus*) supplied by Messrs. Light and Co. Ltd. ¶ Hydrolysis gave αγ-dab monohydrochloride, $[\alpha]_D^{22}$ +20·0°. ‖ Trace. ** Major peak.

EXPERIMENTAL

The following DNP-amino-acids were prepared according to the method of Roa and Sober,[50] αγ-$[DNP]_2$·dab, m. p. 120—122° (from aqueous acetone), DNP·thr, m. p. 145° [from ether–light petroleum (b. p. 60—80°)], and DNP·leu, m. p. 96° [from ether–light petroleum (b. p. 60—80°)].

γ-Dinitrophenyl-αγ-diaminobutyric Acid.—This was prepared by hydrolysing penta-DNP-polymyxin B in a sealed tube for 18 hr. at 100° with 10N-hydrochloric acid–glacial acetic acid (1 : 1, v/v). On concentrating and cooling, γ-DNP·dab monohydrochloride separated and was dissolved in 2N-hydrochloric acid, filtered, and precipitated with pyridine. Crystallisation from aqueous acetone gave yellow prisms, m. p. 260—262° (lit.,[23] 263—264°) (Found: C, 42·4; H, 4·1; N, 19·9. Calc. for $C_{10}H_{12}N_4O_6$: C, 42·3; H, 4·2; N, 19·7%).

α-Dinitrophenyl-αγ-diaminobutyric acid.—L-3-Aminopyrrolidone [51] (0·2 g.) was shaken for 2 hr. with sodium hydrogen carbonate (0·4 g.) and FDNB (0·28 ml.) in 50% aqueous alcohol (15 ml.). The 3-dinitrophenylaminopyrrolidone, m. p. 205—206° (from aqueous alcohol) was

[50] Roa and Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 1328.
[51] Wilkinson, *J.*, 1951, 104.

TABLE 18.

Compound	R_F in solvent * 1	2	3	Electrophoresis † Front (cm.)	Centre (cm.)
α-DNP·dab	0·33	0·53	0·60	−7·7	−7·4
γ-DNP·dab	0·41	0·50	0·65	−6·7	−6·5
αγ-[DNP]$_2$·dab	0·63	0·07 ‡§	0·90	+1·8	+1·5
DNP·leu	0·77 ¶	0·43	Front	+1·8	+1·4
DNP·thr	0·44 ¶	0·51	0·88	+1·8	+1·5
DNPh	0·67	0·32		Sl. +	
DNAn	0·87	0 ‡‖		Sl. +	

* Whatmann No. 1 paper, Solvent 1, n-butanol saturated with water; solvent 2, 1·5M-phosphate buffer, pH 6·0; solvent 3, n-butanol–acetic acid–water (120 : 30 : 50). † Whatmann 3MM paper, 30% aqueous acetic acid, 360v, 7 hr. +, migration to anode; −, migration to cathode. ‡ Streaks. § Vivid yellow fluorescence. ¶ " Biochemists Handbook," ed. Long, E. N. Spon Ltd., London, 1961, gives R_F 0·74 and 0·43, respectively. ‖ Brown fluorescence.

refluxed for 1 hr. with 5N-hydrochloric acid (5 ml.). The solution was cooled, diluted with two volumes of water, and basified with pyridine to give the acid as yellow plates, m. p. 204—205° (lit.,[23] 202°, prepared from L-α-amino-γ-benzamidobutyric acid) (Found: C, 42·1; H, 4·1; N, 19·8%).

The R_F values and electrophoretic mobilities of these derivatives are given in Table 18 from which it can be seen that, by choice of one or other of the procedures, each derivative can be identified. Dinitrophenol and dinitroaniline, breakdown products during the hydrolysis of DNP-amino-acids and -peptides, are included in the Table. The former is readily removed from the products of hydrolysis by sublimation *in vacuo*.[52,53] With the DNP derivatives, the usual precautions of working in the dark were maintained.

Purification of Polymyxin E and Colistin.—The samples of colistin sulphate (Warner–Lambert Research Institute, Morris Plains, New Jersey, Batch W 1979, The Wellcome Research Laboratories assay 7115 u./mg.) and polymyxin E sulphate (W.R.L. batch P62/6/2, 7528 u./mg.) were demineralised by passage through columns of Zeo-Karb 225 at pH 5·0. The eluates were freeze-dried and each of the amorphous products was purified as follows.

The sulphate (5 g.) was dissolved in water (100 ml.) and the base was precipitated at 0° by addition of ammonia solution (10 ml.; *d* 0·88), filtered, washed free from ammonium salts, and dried *in vacuo* at room temperature over phosphorus pentoxide. The base was converted into the naphthalene-2-sulphonate which crystallised from aqueous alcohol. The twice-recrystallised naphthalenesulphonate was dissolved in 20% aqueous alcohol (100 ml.) at 50° and the base was precipitated by addition of 0·88 ammonia solution, filtered, and washed with water. The moist *base* was suspended in water (75 ml.), brought into solution by addition of N-sulphuric acid, reprecipitated with 0·88 ammonia solution (10 ml.) at 0°, filtered, washed free from ammonium salts, and dried at room temperature over phosphorus pentoxide *in vacuo* [Found (colistin base): C, 54·4; H, 8·7; N, 19·4%. Found (polymyxin E base): C, 54·45; H, 8·5; N, 19·0. $C_{53}H_{100}N_{16}O_{13}$ requires C, 54·45; H, 8·55; N, 19·2%].

The naphthalene-2-sulphonates crystallised as *hexahydrates* from aqueous ethanol [Found (colistin): C, 53·15; H, 6·25; N, 9·75; S, 7·1; H_2O, 4·4. Found (polymyxin E): C, 53·15, H, 6·45; N, 9·6; S, 7·4; H_2O, 4·3. $C_{53}H_{100}N_{16}O_{13},5C_{10}H_8SO_3,6H_2O$ requires C, 53·35; H, 6·05; N, 9·65; S, 6·9; H_2O, 4·7%] and gave the anhydrous *salts*, m. p. 206—210° (decomp.), on drying at 100°/0·01 mm. [Found (colistin): C, 56·15; H, 6·65. Found (polymyxin E): C, 55·9; H, 6·4. $C_{53}H_{100}N_{16}O_{13},5C_{10}H_8SO_3$ requires C, 56·0; H, 6·3%].

The pentaorthophosphates of polymyxin B, polymyxin E, and colistin crystallised as nonahydrates from aqueous alcohol containing 1% orthophosphoric acid, and gave the anhydrous salts when dried at 100°/0·01 mm.: *polymyxin B phosphate nonahydrate*, prisms, m. p. *ca.* 205° (decomp.) (Found: C, 36·3; H, 6·9; N, 12·15; P, 7·75; H_2O, 8·25. $C_{56}H_{98}N_{16}O_{13},5H_3PO_4,9H_2O$ requires C, 36·3; H, 7·1; N, 12·1; P, 8·25; H_2O, 8·75%); *polymyxin E phosphate nonahydrate*, prisms, m. p. *ca.* 209° (decomp.) (Found: C, 34·7; H, 6·9; N, 12·5; P, 8·1; H_2O, 8·9. $C_{53}H_{100}N_{16}O_{13},5H_3PO_4,9H_2O$ requires C, 35·0; H, 7·3; N, 12·3; P, 8·4; H_2O, 8·9%); *colistin phosphate nonahydrate*, prisms, m. p. *ca.* 209° (decomp.) (Found: C, 34·95; H, 7·15; N, 11·95; P, 7·95; H_2O, 9·05%).

DNP-Colistin and DNP-Polymyxin E.—(*a*) *Partial substitution products.* The sulphate

[52] Mills, *Biochem. J.*, 1952, **50**, 707.
[53] Biserte, Holleman-Dehove, and Sautiere, *J. Chromatog.*, 1959, **2**, 225.

(0·5 g.) was dissolved in 50% aqueous ethanol (30 ml.) and sodium hydrogen carbonate (0·7 g.) and FDNB (2·5 ml. of a 50 mg./ml. solution in ethanol) were added. The mixture was shaken for 12 hr. in the dark, concentrated *in vacuo*, and the dry residue was dissolved in 85% aqueous formic acid for electrophoretic examination.

(*b*) *Penta-DNP derivatives.* The sulphate (5 g.) was dissolved in water (60 ml.) and sodium hydrogen carbonate (5 g.) and FDNB (12 g. in 120 ml. ethanol) were added. Nitrogen was blown through the mixture which was shaken in the dark for 12 hr. The alcohol was removed *in vacuo*, water (100 ml.) added, and the aqueous extract decanted from the precipitated solid. The solid was dried *in vacuo* at room temperature over phosphorus pentoxide, triturated several times with ether, dissolved in glacial acetic acid (35 ml.), and precipitated from the filtered solution by the addition of 10 volumes of dry ether (yield, 6·8 g.). Electrophoresis [Whatman 3MM paper, 360v, 5 hr., 85% formic acid–acetic acid–water (28 : 30 : 52)] gave no migrating bands and showed that the products were the penta-DNP derivatives.

Acetyl Colistin and Acetyl Polymyxin.—(*a*) *Partial substitution products.* The base (0·1 g.) was dissolved in glacial acetic acid (5 ml.) and acetic anhydride (0·02 ml.) added. After 12 hr. the product was precipitated by the addition of 10 volumes of dry ether.

(*b*) *Fully substituted products.* The base (0·1 g.) was dissolved in glacial acetic acid (9 ml.) and acetic anhydride (1 ml.) added. The optical rotation of the solution increased on standing and finally reached a constant value after about 2 hr. (see Table 11). The product was precipitated by the addition of 10 volumes of dry ether.

Partial Hydrolysates.—(*a*) *DNP-polymyxin E and DNP-colistin.* The penta-DNP derivative (6·5 g.) was dissolved in a mixture of concentrated hydrochloric acid and glacial acetic acid (200 ml.; 1 : 1, v/v) and maintained in a thermostatically controlled bath in the dark at 37° for 6 days. The solution was evaporated under reduced pressure in a rotary evaporator at 37° and the residue was dried *in vacuo* at room temperature over phosphorus pentoxide. The finely powdered amorphous solid was extracted with boiling ethyl acetate (4 × 500 ml.) until no further colour was obtained in the extract. The combined extracts were evaporated under reduced pressure, the somewhat sticky residue was dissolved in 50% aqueous alcohol (300 ml.) and passed down a column of IR-120 H resin, and the column was washed with 300 ml. of the same solvent. The combined eluates were evaporated under reduced pressure, re-evaporated with alcohol, and dried over phosphorus pentoxide (yield, 2·4 g.). When examined electrophoretically the ethyl acetate-soluble and -insoluble fractions gave the bands listed in Table 15.

(*b*) *Polymyxin E and colistin.* The base (200 mg.) was hydrolysed for 6 hr. in concentrated hydrochloric acid (5 ml.) at 37°. The solution was evaporated at the same temperature under reduced pressure and the residual acid was removed by re-evaporating twice with water (10 ml.).

(*c*) *Polymyxin* E_1. The base (2 g.), obtained by countercurrent distribution of polymyxin E, was hydrolysed for 6 days at 37° in concentrated hydrochloric acid (50 ml.). The acid was removed by repeated evaporation with water under reduced pressure at 37°.

Fatty Acids.—(±)-6-Methyloctanoic acid (isopelargonic acid), b. p. 138—140°/14 mm. n_D^{20} 1·4338, was prepared from dihydropyran and s-butyl bromide by the method of Crombie and Harper.[54] (+)-6-Methyloctanoic acid was obtained as a generous gift from Dr. Vogler. (±)-5-Methylheptanoic acid was prepared according to the general method as illustrated by Vogler and Chopard-dit-Jean.[55] Ethyl s-butyl malonate (405 g.; b. p. 110—116°/16 mm.) was hydrolysed by refluxing for 2 hr. with potassium hydroxide (400 g.) in water (400 ml.). After removal of the ethanol by addition and distillation of water (400 ml.), the product was decarboxylated by addition of concentrated sulphuric acid (256 ml.) and refluxing for 3 hr. The 3-methylpentanoic acid was isolated by steam-distillation using an automatic separator.[56] The recovered acid was dried by distillation with benzene and fractionated, b. p. 194—197° (159 g., 73%). 3-Methylpentanol, b. p. 151—152° (81·5%), was prepared by reducing the acid with lithium aluminium hydride, and was converted into 3-methylpentyl bromide, b. p. 140—144° (60%), with phosphorus tribromide and pyridine. Reaction of the bromide with diethyl malonate, hydrolysis at 25° for 24 hr. with aqueous potassium hydroxide, and decarboxylation at 160° for 3½ hr. gave (±)-5-methylheptanoic acid (50·4%), b. p. 125—128°/12 mm., n_D^{20} 1·4290. The *amide*, purified by crystallisation from light petroleum (b. p. 60—80°) and

[54] Crombie and Harper, *J.*, 1950, 2685.
[55] Vogler and Chopard-dit-Jean, *Helv. Chim. Acta*, 1960, **43**, 279.
[56] *Org. Synth.*, Coll. Vol. I, Wiley, New York, p. 413.

sublimation at 60°/0·01 mm., melted at 74—75° (Found: C, 67·4; H, 12·0; N, 9·8. $C_8H_{17}NO$ requires C, 67·1; H, 11·9; N, 9·8%).

(+)-5-*Methylheptanoic acid.* (±)-3-Methylpentanoic acid was resolved by the method of Levene and Marker.[57] (+)-3-Methylpentanoic acid, obtained from the insoluble quinine salt, was distilled at 194—198° and then at 88—90°/12 mm., n_D^{20} 1·4158, $[\alpha]_D^{22}$ +6·24° [*c* 5·7 in light petroleum (b. p. 60—80°)] {cf. b. p. 194—196°, n_D^{25} 1·4158, $[\alpha]_D^{25}$ +6·43° (liquid neat); [58] b. p. 194—196°, n_D^{20} 1·4158, $[\alpha]_D^{22}$ +7·66° (liquid neat); [59] M_D +10°,[60] $[\alpha]_D$ +8·37° [61]}. The *amide* was obtained as colourless plates, m. p. 122—123° (from light petroleum), b. p. 60—80° (m. p. 123° after sublimation at 80°/0·01 mm.) (Found: C, 62·8; H, 11·2; N, 12·0. $C_6H_{13}NO$ requires C, 62·6; H, 11·3; N, 12·2%) and the 4-*bromobenzylthiouronium salt* as colourless plates, m. p. 157—158° (from ethanol) (Found: N, 7·5; S, 8·9. $C_{14}H_{21}BrN_2O_2S$ requires N, 7·75; S, 8·85%). (+)-5-Methylpentanol (b. p. 62°/13 mm.), obtained by reduction of the acid with lithium aluminium hydride, was converted to the bromide (b. p. 146—147°) with phosphorus tribromide and pyridine, and treated with ethyl malonate, to give, after hydrolysis and decarboxylation, (+)-5-methylheptanoic acid, b. p. 125—128°/13 mm., n_D^{20} 1·4289, $[\alpha]_D^{22}$ +6·58 (*c* 3·8 in light petroleum), $[\alpha]_D^{22}$ +7·06° (liquid neat). The *amide*, purified by crystallisation from light petroleum (b. p. 60—80°) and sublimation at 60°/0·01 mm., had m. p. 74° (Found: C, 66·95; H, 11·6; N, 9·5%).

6-*Methylheptanoic acid.* 4-Methylpentanol (b. p. 60°/14 mm.), prepared from 4-methylpentanoic acid by reduction with lithium aluminium hydride, was converted into isohexyl bromide (b. p. 60—62°/14 mm.) with phosphorus tribromide and pyridine. Reaction of the bromide with diethylmalonate, followed by hydrolysis and decarboxylation, gave 6-methylheptanoic acid, b. p. 124—126°/15 mm., n_D^{23} 1·4258. The *amide*, crystallised from light petroleum (b. p. 60—80°) and sublimed at 85°/0·01 mm., had m. p. 110—111° (Found: C, 67·2; H, 12·0; N, 9·5%).

For gas chromatography, the acids were treated with diazomethane in ether and distilled to give methyl (+)-6-methyloctanoate, b. p. 88—90°/14 mm., methyl (+)- and (±)-5-methylheptanoate, b. p. 78—80°/12 mm., and methyl 6-methylheptanoate, b. p. 80°/12 mm.

The fatty acids were obtained from the polymyxins by hydrolysis at 110° for 24 hr. in nitrogen-filled sealed tubes with 5N-sulphuric acid as recommended by Hausmann and Craig,[16] rather than with 6N-hydrochloric acid.

(+)-6-*Methyloctanoic Acid from Polymyxin* E_1.—The acid was obtained as a colourless oil, $[\alpha]_{5461}^{22}$ +8·87° (*c* 7·5 in ether) {cf. synthetic acid, $[\alpha]_{5461}^{22}$ +9·10° (*c* 10 in ether)} (Found: C, 68·4; H, 11·2. Calc. for $C_9H_{18}O_2$: C, 68·35; H, 11·5%). The *amide* had m. p. 91—92° (undepressed on admixture with the synthetic amide) (Found: C, 68·7; H, 12·4; N, 9·1. $C_9H_{19}NO$ requires C, 68·8; H, 12·1; N, 8·9%). The 4-*bromobenzylthiouronium salt* had m. p. 161° (undepressed on admixture with the synthetic salt) (Found: C, 50·5; H, 6·6; Br, 20·1; N, 6·9; S, 7·5. $C_{17}H_{28}BrN_2O_2S$ requires C, 50·6; H, 6·95; Br, 19·9; N, 6·95; S, 7·9%).

6-*Methylheptanoic Acid from Polymyxins* B_2 *and* E_2.—The two acids had $[\alpha]_{5461}^{22}$ 0·0° (*c* = 2·0 in ether) and each gave an optically inactive amide, m. p. 110° (undepressed on admixture with each other and with the amide of 6-methylheptanoic acid but depressed after addition of n-octanamide, m. p. 103—104°). [Found (amide from polymyxin B_2): C, 66·9; H, 11·7; N, 9·8. Found (amide from polymyxin E_2): C, 67·3; H, 12·0; N, 9·5. $C_8H_{17}NO$ requires C, 67·1; H, 11·9; N, 9·8%]. The natural and synthetic acids behaved identically on the gas and paper chromatograms:

Acid	R_F *	M. p. of amide
From polymyxin B_2	0·50	110°
From polymyxin E_2	0·50	110
n-Octanoic acid	0·50	103—104
(+)-5-Methylheptanoic acid	0·50	74—75
6-Methylheptanoic acid	0·50	110
(+)-6-Methyloctanoic acid	0·65	91—92

* Hydroxamates on Whatman No. 1 with benzene–formic acid–water (1 : 1 : 1).

[57] Levene and Marker, *J. Biol. Chem.*, 1931, **91**, 77.
[58] Sax and Bergmann, *J. Amer. Chem. Soc.*, 1955, **77**, 1910.
[59] Sabetay and Panouse, *Compt. rend.*, 1947, **225**, 887.
[60] Klyne, *Biochem. J.*, 1953, **53**, 378.
[61] Marekwald and Nolda, *Ber.*, 1909, **42**, 1583.

The infrared spectra (KCl discs) of the natural and synthetic amides were identical. The methyl deformation bands associated with the isopropyl group appear at 1375 ($\varepsilon \sim 47$) and 1368 cm.$^{-1}$ ($\varepsilon \sim 51$). The methylene rocking modes which appear at 731 cm.$^{-1}$ are to be associated with the relatively short methylene chain. (In n-octanamide and 5-methylheptanamide the band appears at 724 and 773 cm.$^{-1}$, respectively.)

The authors thank Dr. A. J. Everett for the measurements of the spectra, Mr. P. R. W. Baker for the microanalyses, Dr. T. S. G. Jones for the gas-chromatographic studies, and Mr. E. Taylor for technical assistance. Acknowledgments are also made to Dr. K. Vogler, Hoffmann-La Roche & Company, and to Dr. S. H. Harper, King's College, London, for generous samples of fatty acids.

The Wellcome Research Laboratories,
Beckenham, Kent.

[*Received, October 26th*, 1963.]