QUANTITATIVE DETERMINATION OF α- AND γ-2,4-DINITROPHENYL-ISOMERS OF α,γ-DIAMINOBUTYRIC ACID WITH AN AUTOMATIC AMINO ACID ANALYZER AS A METHOD OF STUDYING $N^{\alpha} \rightleftarrows N^{\gamma}$ MIGRATION IN PEPTIDES OF α,γ-DIAMINOBUTYRIC ACID AND POLYMYXIN M

A. B. SILAEV, L. A. BARATOVA AND G. S. KATRUKHA

Laboratory of Chemistry of Proteins, Nucleoproteides and Antibiotics, Chemical Faculty, Moscow State University, Moscow (U.S.S.R.)

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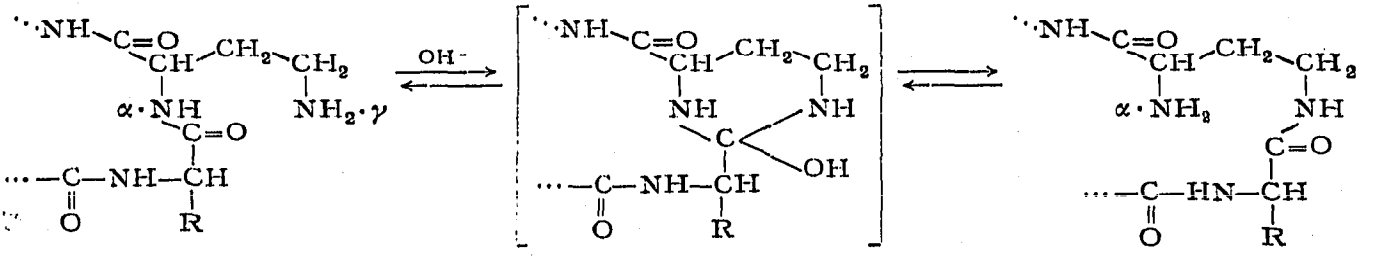
According to the data reported in the literature, salts of antibiotics of the polymyxin group are stable in weak acid and neutral solutions, but are inactivated in acid solutions and in particular in alkaline ones[1–5]. The study of the alkaline inactivation of the antibiotic polymyxin M has shown that in 0.1 *N* NH_4OH at 37° the antibiotic almost completely loses its antibacterial activity in three to five days[6,7]. The inactivation is caused by the change of the secondary structure of the molecule, by a partial racemization of the amino acids forming the molecule of polymyxin M, and finally by intramolecular transfer of the amino acid acyl residue from the α-amino group to the γ-amino group of α,γ-DAB*, which takes place according to the following scheme[8–10].



(I) (II) (III)

The possibility of such $N^{\alpha} \rightarrow N^{\gamma}$ and vice versa $N^{\gamma} \rightarrow N^{\alpha}$ migrations occurring was later qualitatively demonstrated on model peptides[11,12]. The problem confronting us was that of the quantitative estimation of the $N^{\alpha} \rightarrow N^{\gamma}$ migration. To solve it, one had to know the composition of the α-peptide of α,γ-DAB and of the γ-peptide formed as a result of $N^{\alpha} \rightarrow N^{\gamma}$ migration and the quantity of γ-peptide and α-peptide formed from it in the case of $N^{\gamma} \rightarrow N^{\alpha}$ migration. The simplest way to achieve this is by marking the free α- or γ-amino group of the α,γ-DAB forming the peptide with, for example, a DNP-residue. Then the quantity of α-peptide in the mixture may be easily determined from the percentage of γ-DNP-DAB, while the quantity of γ-

Abbreviations: Pel = pelargonic acid; α,γ-DAB = α,γ-diaminobutyric acid; DNP = 2,4-dinitrophenyl-; FDNP = 1-fluoro-2,4-dinitrobenzene; Gly-DAB = glycyl-diaminobutyryl diketopiperazine; DAB-DAB = α,γ-DAB diketopiperazine; Thr = threonine; Leu = leucine.

peptide may be determined from the amount of α-DNP-DAB obtained upon hydrolysis and separation of the DNP-isomers of α,γ-DAB. The main difficulty consists in the separation of the α- and γ-DNP-derivatives of α,γ-DAB. There are only a few, not very effective, ways of separating the α-DNP- and γ-DNP-isomers of α,γ-DAB described in the literature[8,10,13,14]. The most complete separation was achieved by paper electrophoresis at pH 9.1 (ref. 14). However, the analysis takes a long time (17 h) and requires additional correction factors for the adsorption of DNP-derivatives on the paper. In the present study we used an automatic amino acid analyzer for the separation of the α- and γ-DNP-isomers[15,16].

α- and γ-peptides of α,γ-DAB and polymyxin M, after being sustained under conditions of inactivation of the antibiotic (0.1 *N* NH_4OH, 37°, for four days), were subjected to dinitrophenylation in a bicarbonate buffer pH 9.5 according to methods described previously[17]. The completeness of dinitrophenylation was checked by paper electrophoresis in the mixture: 85 % formic acid–glacial acetic acid–water (28:20:52) (300 V, 2–3 h)[18,19]. Then the DNP-peptides, without preliminary refining to prevent losses, were hydrolyzed by 6 *N* HCl for 16 h at 106–108°, and after complete removal of HCl, studied with the "Hitachi" amino acid analyzer, KLA-2 type. The analysis was carried out on a 0.9 × 15 cm column filled with Amberlite CG-120, type III (400 mesh). A 0.30 *N* Na^+-citrate buffer with a pH of 5.28 ± 0.02 was used as eluting buffer. The separation of α-DNP- and γ-DNP-isomers of α,γ-DAB is based on their differences in solubility and basicity. Fig. 1 depicts an elution curve for α- and γ-DNP-DAB, α,γ-DAB and NH_3. The first peak is that of α-DNP-DAB (106 min ± 3 min), the final one (195 min ± 3 min) is the peak of γ-DNP-DAB. The whole analysis takes about 3.5 h.

The clear-cut separation of the pairs α-DNP-DAB and α,γ-DAB, and NH_3 and γ-

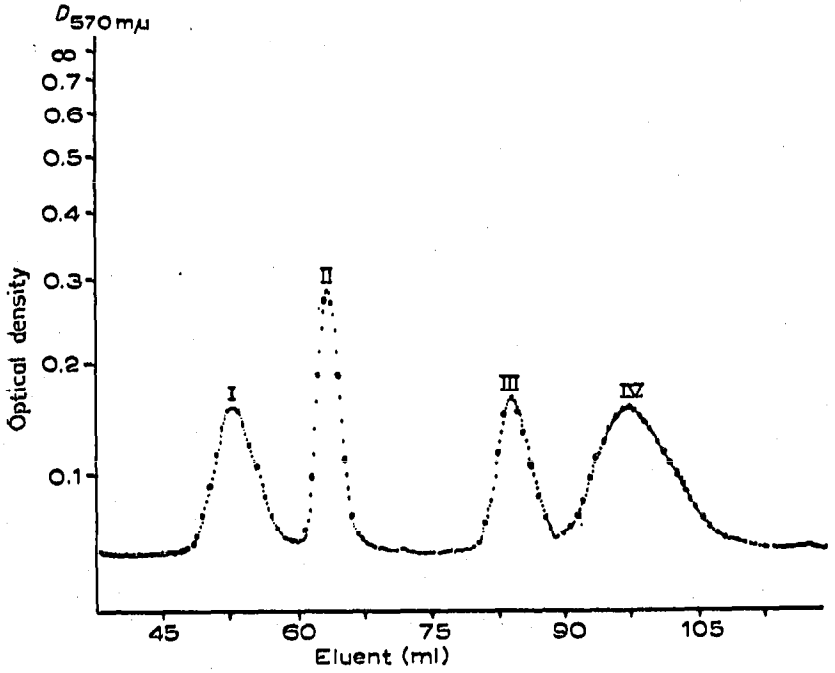


Fig. 1. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.30 *N* Na^+-citrate buffer, pH 5.28 ± 0.02. I = α-DNP-DAB; II = α,γ-DAB; III = NH_3; IV = γ-DNP-DAB.

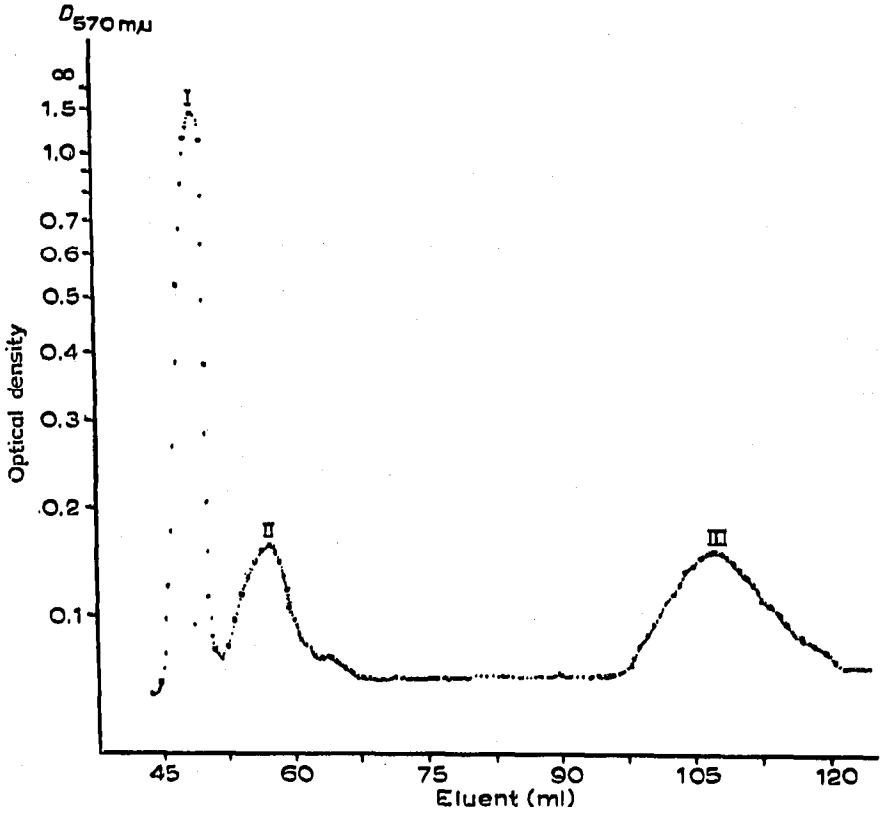


Fig. 2. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.30 *N* Na^{+}-citrate buffer, pH 5.28 ± 0.02. I = Lysine; II = α-DNP-lysine; III = ε-DNP-lysine.

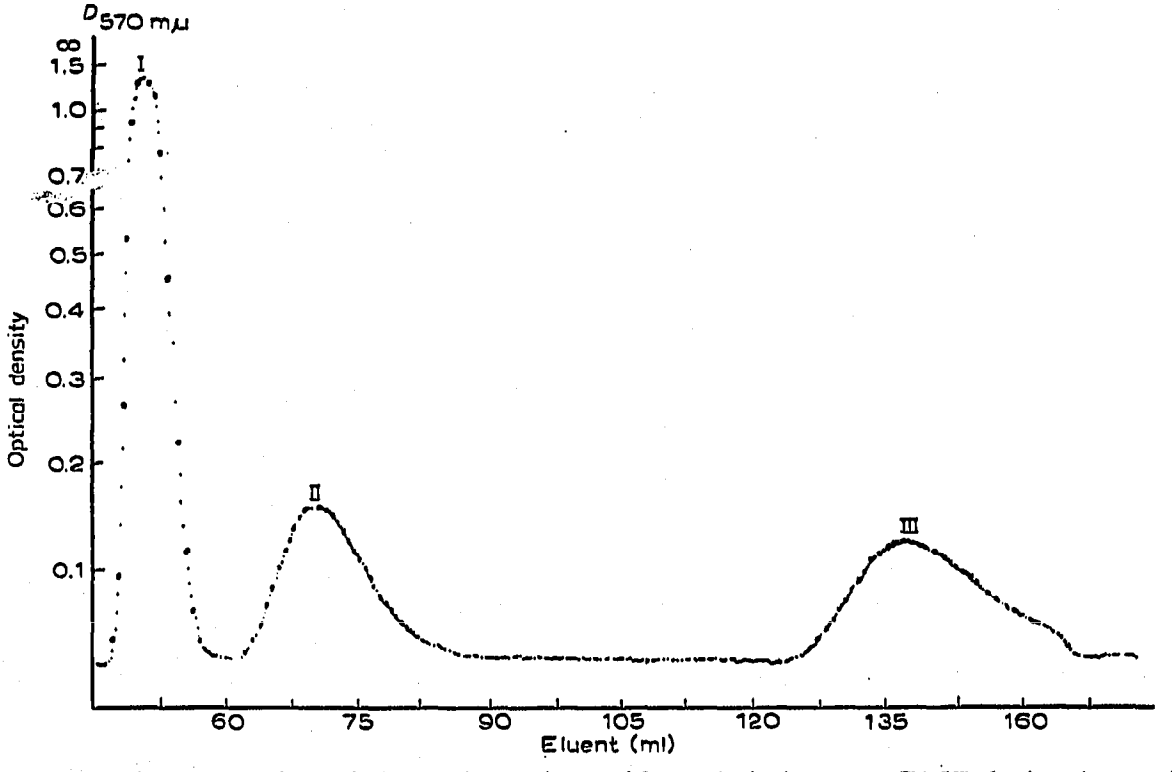


Fig. 3. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.7 *N* Na^{+}-citrate buffer, pH 5.28. I = Ornithine; II = ε-DNP-ornithine; III = δ-DNP-ornithine.

TABLE I

THE RESULTS OF THE QUANTITATIVE ANALYSIS OF PEPTIDES AND OF AN INACTIVE POLYMYXIN M

15-cm column; temperature 50°; eluent 0.30 *N* Na^+-citrate buffer; pH 5.28 ± 0.02.
The analysis of the peptides was carried out after their complete dinitrophenylation and acid hydrolysis under standard conditions. The data presented in the table are given as the degree of breakdown of the DNP-amino acids during hydrolysis: α-DNP-DAB is destroyed to an extent of 16.6% (6 *N* HCl, 16 h, 105–108°), γ-DNP-DAB to an extent of 10.5% (according to data in the literature[13] 10–11%). (a) Before keeping in a thermostat in 0.1 *N* NH_4OH; (b) after keeping in a thermostat in 0.1 *N* NH_4OH for 4 days at 37 ± 1°.

No.	*Compound analysed*		*Amino acid residue*						*Quantity of isomer peptide (degree of re-acylation)*
			α-DNP-DAB		*γ-DNP-DAB*		*α,γ-DAB*		
			μmole	%	*μmole*	%	*μmole*	%	
1	α-Pel-DAB		0.95	56.5	0.65	39.3	0.07	4.2	56.5
2	γ-Pel-DAB		1.12	59.4	0.63	33.5	0.13	7.1	33.5
3	α-Gly-DAB	(a)	—	—	1.05	—	—	—	—
		(b)	2.48	76.8	0.39	11.7	0.43	13.1	76.8
4	γ-Gly-DAB	(a)	1.38	92.8	—	—	0.12	8.0	—
		(b)	0.56	81.7	0.07	9.9	0.06	8.5	9.9
5	Inactive DNP-polymyxin M*		0.341	50.2	0.304	44.8	0.034	5.0	50.2
6	Gly-DAB		0.58	26.1	0.19	8.9	1.43	65.0	—
7	DAB-DAB		0.25	14.1	0.68	38.0	0.86	47.9	—

* In estimating we took into consideration quantities of α-DNP- and γ-DNP-DAB and an excess quantity of α,γ-DAB as compared to the active DNP-polymyxin M (see text p. 65).

DNP-DAB ensures a precise identification and a quantitative estimation according to the absorption at 570 mμ. Under these conditions the micromolar absorption coefficient (*G*) is equal to 6.6 for α-DNP-DAB, 12.6 for γ-DNP-DAB, and 13.1 for α,γ-DAB. It should be mentioned that under identical conditions a clear-cut separation of α-DNP-lysine, ε-DNP-lysine and lysine is also obtained (see Fig. 2). The separation of ornithine and its DNP-isomers is satisfactorily carried out in 0.7 *N* Na^+-citrate buffer pH 5.28 (see Fig. 3). Neutral amino acids and their DNP-derivatives as well as FDNB and dinitrophenol are eluted from the column in the first 50–65 min. Table I shows the results of the analysis of four synthetic peptides* and of two diketopiperazines of α,γ-DAB and of an inactive DNP-polymyxin M.

Table I illustrates that re-acylation occurs in α- as well as in γ-peptides of α,γ-DAB. However, $N^\alpha \rightarrow N^\gamma$ migration goes on more extensively than $N^\gamma \rightarrow N^\alpha$-migration, *i.e.* a shift towards the greater formation and preservation of the γ-peptide is observed. In other words, under conditions of polymyxin M inactivation an equilibrium mixture is formed with 58% of the γ- and 36% of the α-peptide in the case of α- and γ-Pel-DAB, and 79% of the γ- and 10% of the α-isomer in the case of α- and γ-Gly-DAB. This phenomenon may be explained by a lower basicity of the α-amino group of α,γ-DAB as compared to that of the γ-amino group, as well as by a possible steric hindrance of the attack of the —C(O)NH(γ) bond by an α-amino group. In addition, the degree of re-acylation also depends on the kind of acyl residue. Thus, for γ-Pel-DAB $N^\gamma \rightarrow N^\alpha$ migration is 33.5%, whereas for γ-Gly-DAB it is only 9.9%.

* The peptides were synthesized by K. PODUSHKA (ČSSR) and by one of our colleagues[12].

It is noteworthy that in hydrolysates of all the peptides and of an inactive DNP-polymyxin M, inactivated in 0.1 *N* NH_4OH and processed by FDNB, it was possible to find from 4.2 to 13.1 % of free α,γ-DAB upon acid hydrolysis. Control hydrolysates of α-DNP-DAB, γ-DNP-DAB and dinitrophenylated α-Gly-DAB did not yield free α,γ-DAB. However, in the hydrolysate of the DNP-peptide of γ-Gly-DAB, 8 % of free α,γ-DAB was discovered. Its presence may be explained either by the formation of an intermediate substance (II) (see the scheme), during dinitrophenylation, or simply by the breakdown of this peptide in the process of hydrolysis.

The intermediate substance II, exposed to FDNB, upon hydrolysis yields neither α- nor γ-DNP-DAB, but only free α,γ-DAB. Therefore, according to the quantity of the latter it is possible to estimate quantitatively in the reaction mixture the amount of the intermediate substance II formed during the period of inactivation.

The method for the quantitative estimation of α- and γ-DNP-isomers of α,γ-DAB suggested in this paper enables the complete amino acid composition of the DNP-derivatives of active and inactive polymyxin M to be determined and, on this basis, the quantitative estimation of the $N^{\alpha} \rightarrow N^{\gamma}$ migration in the antibiotic during its inactivation. It was found that active DNP-polymyxin M has the following proportions: Leu:Thr:α,γ-DAB:γ-DNP-DAB = 1.0:2.7:0.9:4.9, respectively, which coincides with the data on its structure[20,21]. For an inactive polymyxin M the following proportion of amino acids was obtained: Leu:Thr:α,γ-DAB:γ-DNP-DAB:α-DNP-DAB = 1:2.5:1.2:2.3:2.5, respectively. Thus, an inactivated antibiotic, as well as an active one, contains five free amino groups of α,γ-DAB and the $N^{\alpha} \rightarrow N^{\gamma}$ migration taking place in it is 50 % (see Table I). It is also important that in the case of an inactivated DNP-polymyxin M one may observe the formation of a small excess (5 %) of free α,γ-DAB as compared to the active antibiotic. In our opinion it would be wrong to think that only half of the γ-amino groups took part in re-acylation. In this case $N^{\alpha} \rightarrow N^{\gamma}$ migration in these γ-amino groups should have been 100 %. However, the experiment with the model peptide α-Pel-DAB-demonstrated a 56.5 % migration, whereas with α-Gly-DAB it was 76.8 %.

It is believed that under the conditions described all five free amino groups of the antibiotic take part in $N^{\alpha} \rightarrow N^{\gamma}$ migration. The per cent of re-acylation is an arithmetical mean.

Parallel to the change in the secondary structure and in optical activity, caused by the long-term effect of an alkali medium, the appearance of free α-amino groups of α,γ-DAB and of an intermediate substance II in each molecule of polymyxin M causes a sharp change in its microbiological properties. This fact is worthy of further detailed study.

It is of interest that this method also permitted a quantitative study of isomerization of Gly-DAB and DAB-DAB diketopiperazines, described previously[12]. It was shown that under conditions of inactivation of polymyxin M, as a result of ammonolysis, 53 % of α-DAB-pyrrolidone and 12 % of α-aminopyrrolidone are formed from DAB-DAB, whereas $N^{\alpha} \rightarrow N^{\gamma}$ migration does not take place in diketopiperazine. Gly-DAB yields under these conditions 71 % of α-glycyl-pyrrolidone; only 8 % of the initial diketopiperazine remains.

The quantitative study of $N^{\alpha} \rightarrow N^{\gamma}$ migration in different peptides of α,γ-DAB is being continued.

CONCLUSIONS

A rapid and convenient method of separation and quantitative estimation of α- and ω-DNP-derivatives of α,γ-DAB, ornithine and lysine has been suggested.

A quantitative estimation of the $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in four peptides of α,γ-DAB has been carried out.

The extent of migration in inactive polymyxin M has been determined.

It was shown that in α- and γ-DNP-isomers of N-Pel-DAB, under conditions of inactivation of polymyxin M, a unique mixture is formed which contains about 58 % of the γ- and 36 % of the α-isomer; in N-Gly-DAB peptides, under these conditions, the mixture formed has 79 % of the γ- and 10 % of the α-isomer.

SUMMARY

For a quantitative estimation of the value of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in inactive polymyxin M and in peptides of α,γ-diaminobutyric acid (α,γ-DAB) a rapid method of separation and quantitative determination of the α- and γ-2,4-dinitrophenyl-derivatives of α,γ-DAB is suggested. Their breakdown during acid hydrolysis can be followed with an automatic amino acid analyzer (a 15-cm column, Amberlite CG-120; 0.30 *N* Na^+-citrate buffer, pH 5.28, 50°; 3.5 h). This method enables the α- and ω-2,4-dinitrophenyl-isomers of lysine and ornithine to be separated. The method has also been used to study the $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in four peptides of α,γ-DAB and in inactive polymyxin M. It is shown that in the α- and γ-isomers of N-pelargonyl-DAB, under conditions for inactivation of polymyxin M, a unique mixture is formed, containing about 58 % of the γ- and 36 % of the α-isomers; in peptides of N-glycyl-DAB, the mixture formed has 79 % of the γ- and 10 % of the α-isomer. With polymyxin M $N^{\alpha} \rightarrow N^{\gamma}$ migration is 50 %. It is suggested that in polymyxin M all five γ-amino groups take part in $N^{\alpha} \rightarrow N^{\gamma}$ migration. The quantitative aspect of the transformations of diketopiperazines of α,γ-DAB and of glycyl-DAB, occurring under conditions for inactivation of polymyxin, have also been studied.

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