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Note

Separation of ninhydrin-positive compounds on a single-column amino acid analyzer using lithium buffers

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During a study on the biosynthesis of penicillin[1], culture fluids and mycelial extracts of the mould *Penicillium* were examined with use of a single-column amino acid analyzer equipped with a cation-exchange column and with a lithium buffer gradient system as eluent. Since these analyses revealed the presence of many unidentified compounds, it was necessary to investigate the behaviour of a wide range of known materials. Although several papers[2–9] describe the separation of some amino acids using lithium buffers, extensive standardizations such as those reported for sodium buffers[10–12] are not available. Therefore, the elution behaviour of 145 ninhydrin-positive substances has been compared for two Chromobeads type-B resins of different lot numbers. Since most of these compounds are naturally occurring amino acids, the results should be of interest in the analysis (with lithium buffers) of other complex biological fluids, *e.g.*, human urine or plant extracts.

MATERIALS AND METHODS

Amino acids

Standard solutions of 18 and 38 amino acids (each 2.5 μmole/ml; Technicon, Brussels, Belgium) were used. S-Carbamylcysteine[13], α-aminoadipic acid[14], β-methoxyvaline and allo-O-methylthreonine[15], β-hydroxyvaline[16], hydroxypipecolic acid[17], *threo*- and *erythro*-thiolbutyrine[18] and α-amino-β-ethylvaleric acid[19] were prepared in the laboratory. Mixed disulphides were obtained by bubbling oxygen through alkaline solutions of the thiol compounds. S-Carboxymethyl derivatives were prepared by reaction with iodoacetic acid at pH 7.

Penicillins

The penicilloic acid of isopenicillin N was obtained by alkaline degradation of the penicillin[20]; 6-aminopenicillanic acid (6-APA) was a gift from N.V. Gist-Brocades (Delft, The Netherlands).

Peptides

Reduced and oxidized glutathione were products of Koch-Light Labs. (Colnbrook, Great Britain). The dipeptides L-cystinyl-bis-L-valine and L-cystinyl-bis-D-valine were prepared by a modification of the procedure of Roeske[21,22]. The tripeptides

bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-L-valine and bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-D-valine were synthesized as described elsewhere[22]. The thiol forms of these peptides (LL, LD, LLL and LLD) were obtained by reduction with dithiothreitol (DTT). Bis-δ-(L-α-aminoadipyl)-L-cystine and bis-γ-(L-glutamyl)-L-cystine were prepared by the action of carboxypeptidase A (Koch-Light) on the LLL-tripeptide and oxidized glutathione, respectively; the cysteine forms of these peptides were obtained by reduction with DTT.

Chemicals

All reagents for the preparation of the lithium buffers and the ninhydrin reagent were obtained from E. Merck (Darmstadt, G.F.R.). No filtering of the buffers or of the lithium hydroxide solution was necessary; the former were stored at 4°. Ninhydrin reagent was prepared as described in the Technicon manual[23]. Redistilled deionized water was used throughout.

Chromatographic conditions

The equipment consisted of a Technicon amino acid analyzer with a column (140 × 0.6 cm) filled with Chromobeads Type B (a strongly acidic cation exchange resin) in the lithium form; two batches of this resin (designated as resin I and resin II) were tested. The solutions used in the Autograd are shown in Table I.

TABLE I

GRADIENT FOR THE NINE-CHAMBERED AUTOGRAD

Chamber No.	*Buffer 1 (pH 2.75), ml*	*Buffer 2 (pH 3.01), ml*	*Buffer 3 (pH 6.50), ml*
1	98*	—	—
2	50	50	—
3, 4, 5, 6	—	100	—
7, 8, 9	—	—	100

* Plus 2 ml of isopropyl alcohol.

Buffers of pH 3.01 and pH 6.50 were prepared as described by Vega and Nunn[5]; buffer of pH 2.75 was obtained by acidification (with 6 *M* hydrochloric acid) of the buffer of pH 3.01.

Samples were loaded on the column in 0.20 *M* lithium citrate buffer of pH 2.20; in the presence of thiol compounds, 5–10 mg of DTT were added. Elution was carried out at 37° with a flow-rate of 32 ml/h for 7.45 h, and at 55° with a flow-rate of 40 ml/h for the remainder of the chromatogram (back-pressure 400 p.s.i.). To accelerate elution of arginine, 100 ml of buffer of pH 6.50 was added to chamber No. 9 after 22.15 h. At the completion of each run, the column was washed with 0.3 *M* LiOH for 2 h at 70° and regenerated at the same temperature with buffer of pH 2.75 for 1 h.

RESULTS AND DISCUSSION

Since this work was originally intended for the separation of precursors of penicillin, which are mostly acidic peptides containing α-aminoadipic acid, the elution

system was adapted for optimal resolution in the first part of the chromatogram. The gradient of Vega and Nunn[5] was altered in three ways: 100 ml of buffer was placed in each chamber of the Autograd, a third buffer of pH 2.75 was used, and the content of the pH 6.50 buffer was lowered. For the same reason, the temperature was kept at 37° for 7.45 h, then increased to 55°.

The positions of all 145 ninhydrin-positive compounds on the chromatogram of resin I are shown in Fig. 1; the numbers associated with the various peaks refer to the compounds listed in Table II. As compared with the system of Vega and Nunn[5], the elution orders of cystine and valine, of phenylalanine and β-alanine and of the cystathionines and methionine are reversed; also, the total analysis time up to arginine is lengthened to over 25 h.

A number of compounds not tested by Vega and Nunn[5] were well separated. Allo-γ-hydroxyglutamic acid was eluted before allo-β-hydroxyglutamic acid. The three amino sugars tested (glucosamine, mannosamine and galactosamine) were well resolved. In addition, good separations were obtained for most of the diastereo-

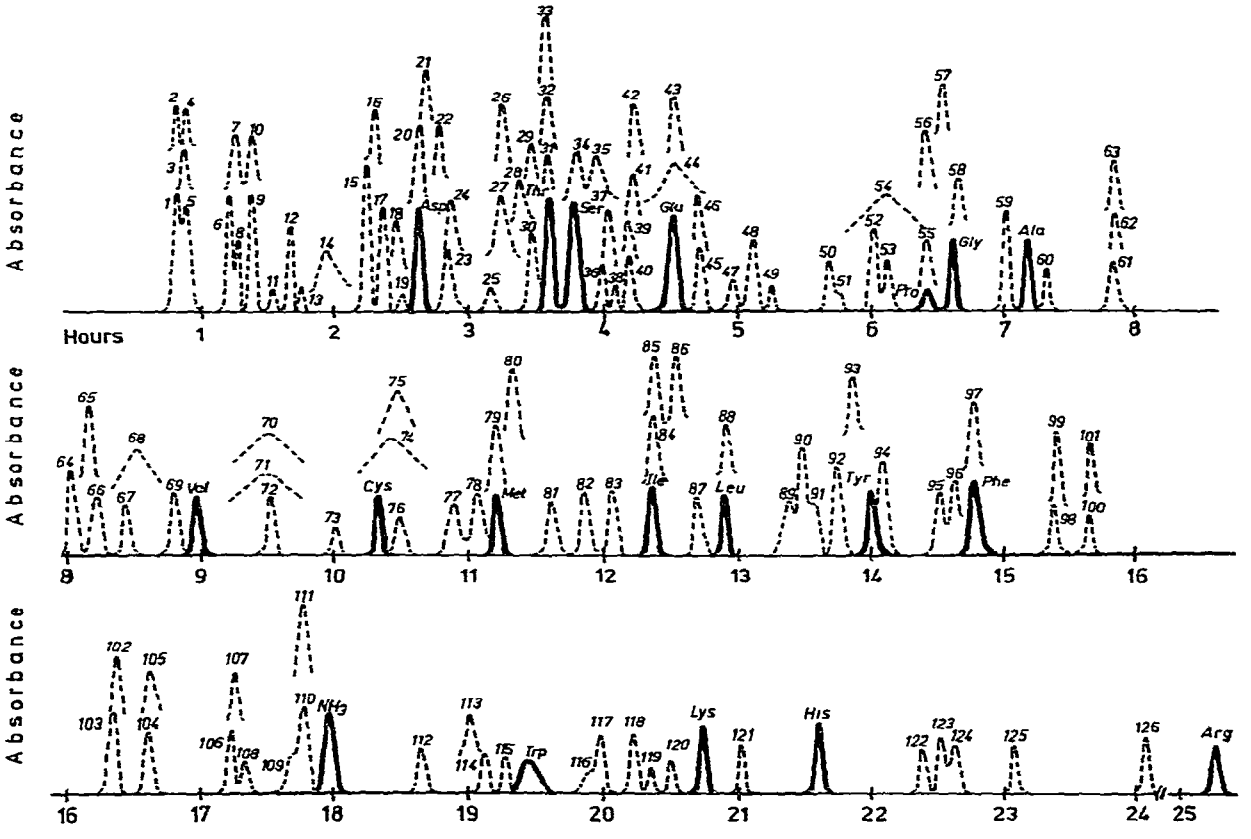

Fig. 1. Elution positions of 145 ninhydrin-positive compounds on resin I. The 18 physiological amino acids, and ammonia, are indicated by solid lines. For identification of numbered peaks (broken lines) see Table II.

TABLE II

IDENTIFICATION OF PEAKS IN FIG. 1

Peak No.	*Compound*	*Peak No.*	*Compound*
1	Cysteic acid	50	Cysteine
2	Homocysteic acid	51	*threo*-Thiolbutyrine
3	Cysteinesulphinic acid	52	S-Methylcysteine
4	O-Phosphothreonine	53	α-Aminoadipic acid
5	O-Phosphoserine	54	Glutathione (oxidized)
6	Taurine	55	*erythro*-Thiolbutyrine
7	Penicillaminic acid	56	S-Carboxymethylhomocysteine
8	*threo*-β-Hydroxyaspartic acid	57	β-Hydroxyleucine
9	Phosphoethanolamine	58	Penicillamine (reduced)
10	Levulinic acid	59	Isoserine
11	Dithiothreitol	60	Lanthionine (peak 1)
12	*erythro*-β-Hydroxyaspartic acid	61	Citrulline
13	Urea	62	Lanthionine (peak 2)
14	S-Carboxymethylglutathione	63	α-Aminoisobutyric acid
15	Allo-γ-hydroxyglutamic acid	64	Glucosamine
16	S-Methylcysteine sulphoxides	65	S-Ethylcysteine
17	Allo-β-hydroxyglutamic acid	66	α-Aminobutyric acid
18	Cephalosporin C	67	Mannosamine
19	3-Hydroxypipecolic acid	68	Bis-γ-(L-glutamyl)-L-cystine
20	S-Carboxymethylcysteine	69	Galactosamine
21	S-Carboxymethylpenicillamine	70	Bis-δ-(L-α-aminoadipyl)-L-cystine
22	Diaminosuccinic acid (peak 1)	71	Bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-L-valine
23	Glutathione (reduced)	72	α-Aminopimelic acid
24	S-Methylglutathione	73	Pipecolic acid
25	4-Hydroxyproline	74	Bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-D-valine
26	Diaminosuccinic acid (peak 2)	75	6-Aminopenicillanic acid
27	Penicilloic acid of isopenicillin N	76	Homocysteine
28	γ-(L-Glutamyl)-L-cysteine	77	Phenylglycine
29	Methionine sulphoxide (peak 1)	78	Homocitrulline
30	Methionine sulphone	79	Norvaline
31	Methionine sulphoxide (peak 2)	80	Mixed disulphide of L-cysteine and D-penicillamine
32	Allo-threonine		
33	β-Hydroxyvaline	81	Allo-isoleucine
34	δ-(L-α-Aminoadipyl)-L-cysteine	82	Ethionine
35	δ-(L-α-Aminoadipyl)-L-cysteinyl-L-valine	83	Djenkolic acid
		84	Penicillamine (oxidized)
36	O-Methylthreonine	85	Cystathionine
37	O-Methylserine	86	Allo-cystathionine
38	Allo-4-hydroxyproline	87	α-Amino-β-hydroxybutyric acid
39	Muramic acid	88	3,4-Dihydroxyphenylalanine
40	Asparagine	89	Isoglutamine
41	Allo-4-hydroxypipecolic acid	90	α,ε-Diaminopimelic acid
42	S-Carbamylcysteine	91	Norleucine
43	β-Methoxyvaline	92	Cycloserine
44	δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine	93	α-Amino-β-ethylvaleric acid
		94	Mixed disulphide of L-cysteine and DL-homocysteine
45	Glutamine		
46	Homoserine	95	β-Alanine
47	4-Oxopipecolic acid	96	Mixed disulphide of DL-homocysteine and D-penicillamine
48	Sarcosine		
49	5-Hydroxypipecolic acid	97	O-Benzylserine

TABLE II *(continued)*

Peak No.	Compound	Peak No.	Compound
98	β-Aminoisobutyric acid	112	δ-Aminovaleric acid
99	δ-Aminolevulinic acid	113	Valinol
100	L-Cysteinyl-L-valine	114	5-Hydroxylysine
101	L-Cysteinyl-D-valine	115	Allo-5-hydroxylysine
102	Argininosuccinic acid	116	Creatinine
103	Homocystine	117	α,γ-Diaminobutyric acid
104	γ-Aminobutyric acid	118	Ornithine
105	S-Benzylcysteine	119	Valinamide
106	5-Hydroxytryptophan	120	ε-Aminocaproic acid
107	α-Aminocaprylic acid	121	1-Methylhistidine
108	Ethanolamine	122	3-Methylhistidine
109	Kynurenine	123	Carnosine
110	L-Cystinyl-bis-L-valine	124	Homocarnosine
111	L-Cystinyl-bis-D-valine	125	α-Amino-β-guanidinopropionic acid
		126	Homocysteine thiolactone

isomers of amino acids and peptides tested: *e.g.*, *threo*- and *erythro*-β-hydroxyaspartic acid; hydroxyproline, isoleucine, 5-hydroxylysine and their allo-forms; *threo*- and *erythro*-thiolbutyrine and the LLL- and LLD-isomers of oxidized and reduced δ-(α-aminoadipyl)-cysteinylvaline. However, some of the compounds were not resolved in our system. The strongly acidic amino acids (cysteic acid, homocysteic acid, phosphoserine and phosphothreonine) always eluted with the front. Identical elution times were obtained for threonine and allo-threonine, for aspartic acid and S-carboxymethylcysteine, for the second peak of lanthionine and α-aminoisobutyric acid, for methionine and norvaline, for 3,4-dihydroxyphenylalanine and leucine, and for β-aminoisobutyric acid and δ-aminolevulinic acid. The LL- and LD-epimers of cysteinylvaline and of cystinyl-bis-valine always ran together.

The elution pattern with resin II differed from that with resin I in several respects. Reduced glutathione was eluted after aspartic acid on resin I, but the two were only marginally separated on resin II. Methionine sulphone and threonine were only separated with resin I. The resolution of δ-(L-α-aminoadipyl)-L-cysteine and the LLL-thiol tripeptide was better with resin I than with resin II. Glutamic acid and glutamine were well resolved on resin I, but had the same retention time on resin II. In contrast, the LLD-thiol peptide and glutamic acid were only separated on resin II. Also, separations of cystine and 6-APA, allo-isoleucine and ethionine, and 3-methylhistidine and carnosine were better on resin I, whereas those of citrulline and lanthionine (peak 2), galactosamine and valine, homocitrulline and methionine, α,ε-diaminopimelic acid and norleucine, and O-benzylserine and phenylalanine were better on resin II. The differences between the two resins remained constant, although both columns were re-packed several times. Differences in separation on the two resins can be advantageous for the identification of unknown compounds; in this way, glutathione and the LLD-thiol tripeptide (labelled with sulphur-35) could be detected in cultures of *Penicillium chrysogenum*[1].

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