ION-EXCHANGE CHROMATOGRAPHY OF SULFUR AMINO ACIDS AND THE SEPARATION OF DIASTEREOISOMERS*

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Cysteine (CySH) and glutathione (GSH) have been shown to participate in several reactions involving conjugation with other organic compounds. Identification of nitrogenous compounds excreted in the urine has been accelerated by chromatographic techniques, most recently by ion-exchange column chromatography as refined in the automatic recording apparatus of SPACKMAN, STEIN AND MOORE[1].

These authors have described the behaviour under ion-exchange chromatography of methionine sulfone, methionine sulfoxides, cysteic acid, S-carboxymethylcysteine, homocystine, felinine, cystathionine, and GSSG[1,2]. HAMILTON AND ANDERSON have noted the chromatographic behavior of DL + *meso*-lanthionine in a similar system[3]. FRIMPTER AND BASS recorded the position of S-aminoethylcysteine, L-2-thiolhistidine, S-methyl-cysteine, S-ethyl-cysteine, S-*n*-butyl-L-cysteine, S-carboxyl-L-cysteine, L-ethionine, D-penicillamine, L-djenkolic acid, and the optical isomers of the disulfide of cysteine and homocysteine, all in the system of SPACKMAN, STEIN AND MOORE[4]. The partial separation of *meso*-cystine from racemic cystine was noted by HIRS, MOORE AND STEIN[5].

The present study was undertaken to confirm the presence in urine of compounds demonstrated by other methods[6], and to describe the chromatographic location of compounds which might be found under certain circumstances in biological fluids or in hydrolysates of synthetic peptides. The chromatographic separation of diastereoisomers of several sulfur compounds is of particular interest because it affords a means of resolving them. In general, the compounds were prepared in the laboratory in Japan, and the chromatographic studies were performed in the laboratory in the United States.

METHODS

Compounds

The compounds investigated are numbered according to their order of elution from the resin column. Compound 5 was purchased from the California Corp. for Biochemical Research, Los Angeles. Compounds 4 ("isovalthine"), 7, 9 ("isobuteine"), 10, 12, 13 were synthesized according to methods previously described[6,7]. Compounds 2, 3, 6 and 8 were synthesized according to the methods described by several authors[8–11]

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respectively. Compound I was prepared by reaction of reduced glutathione with (±)-α-bromoisovaleric acid in triethanolamine solution. Although Compound I was not pure, it yielded isovalthine, glutamic acid, and glycine after hydrolysis in 2 *N* HCl. Its chromatographic behavior on paper will be reported elsewhere[12]. Compound II was synthesized as usual by reaction of L-cysteine with β-bromo-α,α-dimethylpropionic acid in liquid ammonia. Elemental analysis of the Compound II was correct and its R_F values on paper have been reported in a previous paper[7]. β-Bromo-α,α-dimethylpropionic acid was prepared according to reported methods[13,14].

Chromatography

Ion-exchange chromatography was carried out essentially according to the method recommended by SPACKMAN, STEIN AND MOORE for analysis of physiological fluids, that is, at 30°, pH 3.23 with change to 50° and pH 4.25 (0.2 *N* sodium citrate buffers) after 350 effluent ml.

RESULTS

A composite tracing showing the location of the various compounds examined is shown in Fig. 1. Ninhydrin color at 570 mμ, relative to leucine, is listed in Table I.

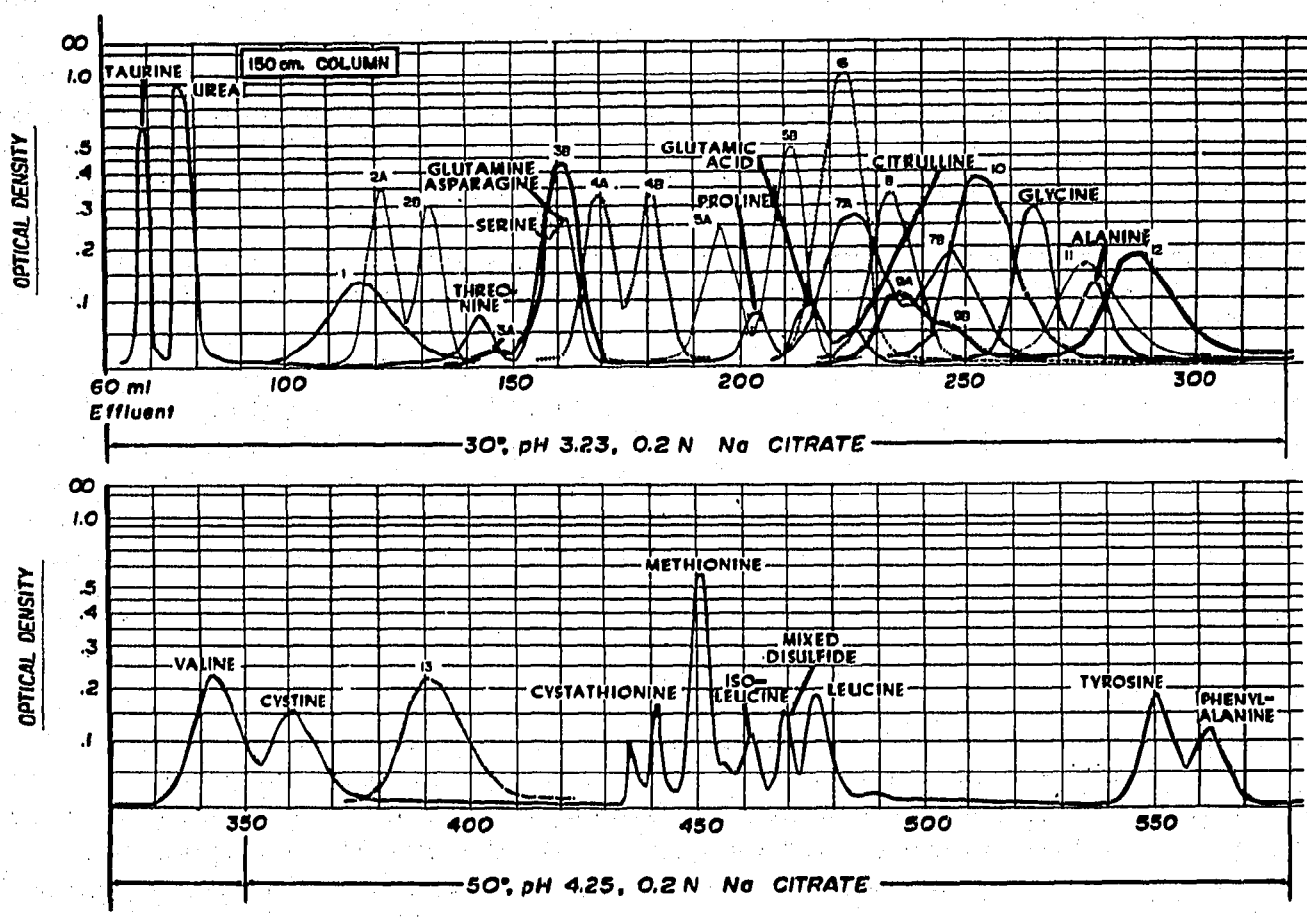

Fig. 1. Composite tracing of ion-exchange chromatogram. The substances which might be found in biological fluids are traced in solid black lines. The numbered peaks correspond to those in Table I.

The chromatographic runs were generally performed once, so that the color value listed is only approximate.

TABLE I

Figure location	*Formula*	*Per cent of leucine color (570 mμ)*
1	GS–CH(COOH)$CH(CH_3)_2$	impure
2 (A + B)	CyS–$CH(CH_3)$COOH	91
3 (A + B)	CyS–$C(CH_3)_2$COOH	impure
4 (A + B)	CyS–CH(COOH)$CH(CH_3)_2$ (isovalthine)	87
5 (A + B)	CyS–$CH_2CH(NH_2)$COOH (lanthionine)	102
6	CyS–CH_2CH_2COOH	100
7 (A + B)	CyS–CH(COOH)$CH_2CH_2CH_3$	92
8	CyS–$CH(CH_3)CH_2$COOH	76
9 (A + B)	CyS–$CH_2CH(CH_3)$COOH (isobuteine)	—
10	CyS–$C(CH_3)_2CH_2$COOH	95
11	CyS–$CH_2C(CH_3)_2$COOH	82
12	CyS–$CH(CH_3)CH(CH_3)$COOH	—
13	Cys–$CH_2CH(CH_3)CH_2$COOH	79

DISCUSSION

In general, the three factors that appear to contribute to delay elution of the CySH conjugates are: increased distance of the carboxyl group from the S, the presence of side chains, and increased molecular weight. The early appearance of Compound 1 is consequent to the presence of 3 carboxyl groups.

Compounds 1, 6, 8, 10, 11, 12 and 13 yielded single peaks suggestive of a "pure" amino acid. The formulas of Compounds 6, 10 and 11 reveal no opportunity for diastereoisomerism. The breadth of peaks from Compounds 1, 12 and 13, whose formulas suggest opportunity for diastereoisomerism, lead to the conjecture that they may represent poorly separated isomers.

Compounds 2, 4, 5, 7 and 9 revealed double, sharp peaks characteristic of diastereoisomers. Lanthionine, 5, probably is separated into *meso*- and DL-forms, which were stated to be present by the manufacturer. Inspection of formulas of the other compounds yielding 2 peaks, *i.e.*, 2, 4, 5, 7 and 9, reveals that all have two potentially asymmetrical carbon configurations. In all of these, one asymmetrical carbon would be at the alpha carbon of CySH and the other at the carbon atom adjacent to the carboxyl of the conjugated acid. Compound 3 produced two peaks which were not typical of those shown by diastereoisomers, and the compound does not contain two asymmetrical carbon atoms. It is concluded, therefore, that the smaller of the two peaks must represent some ninhydrin-positive contaminant.

All compounds yielding paired peaks (except lanthionine) were synthesized by reaction of L-CySH with the appropriate (±)-bromo acid. This suggests the presence of L- and L-*allo*-amino acids.

No definite statement can be made as to which isomer constitutes which peak, since the column effluent was not analyzed. Sufficient amounts were not obtained for optical rotation determinations. Although the resolution of isovalthine diastereo-

isomers was somewhat improved by operation at 50°, increasing the load on the column tended to cause loss of resolution. Chromatography of isovalthine isomers on a 165 × 2 cm "preparative" column of amberlite IR-120 at 50° with pH 3.23, 0.2 *N* sodium citrate buffer did not produce adequate resolution. *meso*-Cystine is eluted prior to DL-cystine in this system[1,4,5]. When cystathionine is chromatographed in this technique, but the shift to pH 4.25 buffer is not made and elution is continued with pH 3.23 buffer[15], *allo*-cystathionine is eluted prior to LL-cystathionine at 515 ml. *allo*-Isoleucine precedes isoleucine[1,2]. L-*allo*-Isovalthine prepared by reaction of L-cysteine with freshly prepared (—)-α-bromoisovaleric acid in triethanolamine solution is eluted prior to L-isovalthine prepared by reaction of L-cysteine with fresh (+)-α-bromoisovaleric acid*. Because the *allo*- or *meso*-form of these four compounds precedes the "racemic" or L-(+) form, it is assumed that this is also the case with the remaining isomers. This may offer a practical method for the separation of chromatographic amounts of diastereoisomers.

A small peak in the area of isovalthine (Compound 4) was observed upon ion-exchange chromatography of the urine of a three-week old cretin. However, the amount present was too small to permit chemical confirmation. It may be noted, however, that the possible presence of some of the compounds in biological fluids would be obscured by usually occurring compounds, *e.g.*, Number 11 by alanine.

SUMMARY

The technique of SPACKMAN, STEIN AND MOORE[1] was used to chromatograph some sulfur-containing amino acids. The appearance of double peaks indicated the probable separation of diastereoisomers.

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* The detailed method of resolution of (±)-α-bromoisovaleric acid to each antipode by using ephedorine will be published.