Separation of proline and hydroxyproline derivatives by thin-layer chromatography

The recent publication of MYHILL AND JACKSON[1] concerning the separation of proline and 4-hydroxyproline N-nitroso derivatives prompted us to report similar results obtained with other derivatives. This note will summarize the thin-layer chromatographic separation of N-nitroso derivatives and of carboxy-*n*-butyl esters of the following imino acids: (a) proline, (b) 3,4-dehydroproline, (c) 3-hydroxyproline (*cis*-form), (d) 3-hydroxyproline (*trans*-form), (e) 4-hydroxyproline, (f) 4-allo-hydroxyproline*.

Procedure

n-Butyl esters. The hydrochlorides of the imino acids (0.2–0.5 mg each) were esterified with 10 ml of *n*-butanol in the presence of a strong cationic resin (Amberlyst 15, kindly obtained from Rohm and Haas Co.). The mixture was gently rotated at 120° for one hour. The ratio between imino acids and resin was 1:20 (w/w). After esterification *n*-butanol was decanted and the resin was filtered on glass under slight vacuum. To the dry resin was then added 10 ml of benzene, containing 0.2 ml of *n*-butylamine; the mixture was refluxed at 70–80° for 15 min.

The resin was discarded and the benzenic solution containing the *n*-butyl esters of the imino acids was concentrated under vacuum (22 mm Hg) in an ice bath.

N-Nitroso derivatives. Nitrous acid (1 ml/mg of imino acid) was added to the mixture of imino acids (0.2–0.5 mg each) at 100° until change of the colour. In order to avoid the destruction of 3,4-dehydroproline the nitrous acid must be prepared from sodium nitrite and acetic acid instead of the usual mixture of sodium nitrite and hydrochloric acid. The N-nitroso derivatives of imino acids were then concentrated under vacuum.

Thin-layer chromatography. Kieselgel G (Merck Co.) was mixed with distilled water (1:2, w/v), stratified with an automatic apparatus (previously described[2]) on glass plates (20 × 20 cm) and heated for one hour at 100–105°.

The amounts of imino acid derivatives deposited were from 10 to 50 μg in volumes of 10–50 μl.

The solvent for *n*-butyl esters was benzene-*n*-butanol (75:25, v/v), and for *N*-nitroso derivatives *n*-butanol–acetic acid–water (120:30:50, v/v).

The substances were visualized with ninhydrin in *n*-butanol saturated with water (0.2 %) or with isatin (0.2 %) in *n*-butanol containing 5 % acetic acid followed by *p*-dimethylaminobenzaldehyde (1 %).

Results

The R_F values obtained for the two series of imino acids with the two staining procedures are reported in Tables I and II.

Good separations of the *n*-butyl esters of imino acids were achieved. This last procedure has been applied with satisfactory results to samples of bovine serum albu-

* Compounds (a), (e) and (f) were obtained from Mann Co., (b) was kindly given by Dr. B. WITKOP, N.I.H., Bethesda, Md., (c) and (d) were kindly given by Dr. D. OGLE, University of Cincinnati.

TABLE I

R_F VALUES OF *n*-BUTYL ESTERS OF IMINO ACIDS

Compound	R_F	*Colour developed with ninhydrin*
n-Butylproline	0.14	yellow-brown
n-Butyl-3,4-dehydroproline	0.55	violet
n-Butyl-3-hydroxyproline (*cis*-)	0.24	yellow
n-Butyl-3-hydroxyproline (*trans*-)	0.10	yellow
n-Butyl-4-hydroxyproline	0.59	yellow
n-Butyl-allo-4-hydroxyproline	0.47	yellow

The solvent used was benzene–*n*-butanol (75:25). No colour appeared with isatin or dimethyl-aminobenzaldehyde spray.

TABLE II

R_F VALUES OF IMINO ACIDS AND THEIR NITROSO DERIVATIVES

Compound	R_F	*Colour developed with*		
		Ninhydrin	*Isatin*	*p-Dimethyl-aminobenzaldehyde*
Proline	0.28	orange	blue	yellow
NO_2-Proline	0.27	yellow	blue	blue
3,4-Dehydroproline	0.29	yellow-green	—	violet
NO_2-3,4-Dehydroproline	0.48	yellow	—	violet
3-Hydroxyproline (*cis*-)	0.29	pink	—	pink
NO_2-3-Hydroxyproline (*cis*-)	0.28	yellow	—	pink
3-Hydroxyproline (*trans*-)	0.23	pink	—	pink
NO_2-3-Hydroxyproline (*trans*-)	0.23	yellow	—	pink
4-Hydroxyproline	0.29	orange	—	pink
NO_2-4-Hydroxyproline	0.28	yellow-pink	—	pink
4-Allo-hydroxyproline	0.23	orange	—	yellow
NO_2-4-Allo-hydroxyproline	0.23	yellow	—	pink

The solvent used was *n*-butanol–acetic acid–water (120:30:50).

min and gelatin after hydrolysis. The nitroso derivatives of the various imino acids on the contrary cannot be easily separated under the experimental conditions of the present work.

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1 D. MYHILL AND D. S. JACKSON, *Anal. Biochem.*, 6 (1963) 193.
2 F. MARCUCCI AND E. MUSSINI, *J. Chromatog.*, 11 (1963) 270.

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