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MECHANISM OF CONVERSION OF SECONDARY AMINES TO PRIMARY AMINES BY SODIUM HYPOCHLORITE

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The mechanism of the conversion of secondary amines by sodium hypochlorite (NaOCl) to compounds that form fluorescent adducts with o-phthalaldehyde (OPA)/2-mercaptoethanol (ME) was investigated by analyzing the reaction mixtures of secondary amines and NaOCl by high performance liquid chromatography (HPLC) and high performance thin-layer chromatography (HPTLC). Methylamine was found to be produced from N-methylamino acids, L-epinephrine, DL-metanephrine and N-methylcyclohexylamine. While N-phenylbenzylamine and N-alkylbenzylamines gave aniline and the corresponding alkylamines, respectively, N-alkylanilines produced the corresponding alkylamines rather than aniline. α-Amino acids were converted to ammonia by NaOCl. On the basis of these findings, mechanism of the conversion of secondary amines to primary amines by NaOCl were proposed.

The postcolumn conversion of secondary amines to primary amines by sodium hypochlorite (NaOCl) or chloramine-T (sodium N-chloro-p-toluenesulfonamide) has been widely employed in high performance liquid chromatographic (HPLC) analyses of proline, hydroxyproline[1)-5)], non-protein cyclic imino acids[6)] and spectinomycin[7)], in combination with the successive fluorescence derivatization with o-phthalaldehyde (OPA)/2-mercaptoethanol (ME) reagent[8)9)]. We found that various classes of secondary amino compounds other than those described above are also converted by NaOCl to compounds detectable with the OPA/ME reagent, which led to the development of the fluorometric method[10)] and the HPLC method[11)] with the fluorescence detection for the determination of secondary amino compounds. Despite the practical value and the promising versatility of the derivatization with NaOCl or chloramine-T, neither the reaction products nor the reaction mechanism has been elucidated so far, though a related work has been reported by Weigele et al.[12)]. In their report on the fluorometric assay of proline and hydroxyproline with fluorescamine, Weigele et al.[12)]

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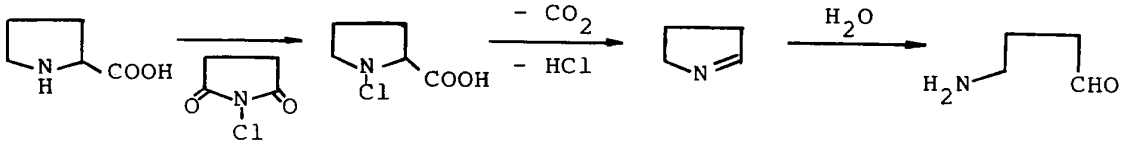
proposed a mechanism of the conversion of proline to γ-aminobutyraldehyde by N-chlorosuccinimide, which involved the initial formation of N-chloroproline, loss of carbon dioxide and hydrogen chloride to produce Δ^1-pyrroline and finally hydrolysis of the cyclic imine to yield the primary amine (Scheme 1).



(Scheme 1)

In the present work, the mechanism of the conversion of various classes of secondary amines by NaOCl to primary amines was investigated by analyzing the reaction mixtures of them by HPLC and high performance thin-layer chromatography (HPTLC).

EXPERIMENTAL

Reagents and materials

The packed columns of Partisil-10 SCX (250 mm × 4.6 mm I.D., 10 μm) and TSK LS-410 (300 mm × 4 mm I.D., 5 μm) were purchased from Whatman (Clifton, NJ, U.S.A.) and Toyo Soda (Tokyo, Japan), respectively. Antiformin (0.7 M NaOCl solution when assayed by iodometry), solvents and reagents for preparing buffers were obtained from Kanto Chemical (Tokyo, Japan). o-Phthalaldehyde (Funa®-phthal) was purchased from Funakoshi Pharmaceutical (Tokyo, Japan). DL-Metanephrine hydrochloride, DL-normetanephrine hydrochloride, N-methyl-L-leucine (Sigma Chemical, St. Louis, MO, U.S.A.), L-proline, L-4-hydroxyproline, DL-alanine, L-aspartic acid, Brij-35 (Nakarai Chemicals, Kyoto, Japan), ME, 2,2'-thiodiethanol (TDE), sarcosine, L-epinephrine bitartrate, L-norepinephrine bitartrate, methylamine hydrochloride, N-methylbenzylamine, N-ethylbenzylamine, N-phenylbenzylamine, N-methylaniline and N-ethylaniline (Tokyo Kasei, Tokyo, Japan) were used as received. Silica gel 60 HPTLC plates (without fluorescent indicator, 10 × 10 cm) were purchased from E. Merck (Darmstdt, G.F.R.).

Preparation of solutions

Ten millimolar solutions of amino compounds were prepared with 20 mM citrate buffer (pH 3.0). The buffer was used to dilute the solutions. The OPA/ME reagent used was the same as described previously[11].

Preparation of reaction mixtures of secondary amines and NaOCl

Fifty microliters of 10 mM amine solution and 500 μl of 7 mM NaOCl in 1 M sodium hydroxide solution were mixed. After allowing the reaction mixture to stand at 60°C for 30 s, 500 μl of 0.2 M TDE solution in 5.7 %(v/v) acetic acid was added to destroy the excess amount of NaOCl[10] followed by thorough mixing. A 5-μl aliquot of the resultant solution was injected to the HPLC system described below.

Preparation of reaction mixtures of primary amino acids and NaOCl

Fifty microliters of 10 mM amino acid solution and 500 μl of 7 mM MaOCl in 0.1 M phosphate buffer (pH 8.0) were mixed and allowed to stand at 40°C for 2min. After adding 500 μl of 0.2 M TDE solution in 0.1 M phosphate buffer (pH 8.0) and

thorough mixing, a 5-µl aliquot of the reaction mixture was analyzed with the HPLC system described below from which the pumps and the tubings for NaOCl and TDE were omitted.

Postcolumn HPLC derivatization system for primary and secondary amines

The flow diagram of the HPLC system used for the analyses of the reaction mixtures of secondary amines and NaOCl is shown in Fig.1. The reaction mixtures were separated on a column of Partisil-10 SCX by isocratic elution with 0.1 M citrate buffer (pH 2.8) delivered through a Mini-micro pump P_1 (Type KHD-26; Kyowa Seimitsu, Tokyo, Japan) at a flow rate of 0.70 ml/min. Some experiments were performed with a column of TSK LS-410 by using 0.1 M citrate buffer (pH 3.0)-methanol (100:10) as eluent (0.70 ml/min). The eluate was mixed in a three-way tee with 0.34 mM NaOCl in 1 M hydroxide solution delivered through a Mini-micro pump P_2 (Type KSD-16; Kyowa Seimitsu) at a flow rate of 0.55 ml/min. The mixture was incubated in the conversion coil C_1 (2 m × 0.5 mm I.D.) at 60°C and mixed in the second three-way tee with 20.2 mM TDE solution in 5.7 % (v/v) acetic acid delivered at a flow rate of 0.53 ml/min through a Mini-micro pump P_3 (Type KHD-16; Kyowa Seimitsu). The outlet of the second tee was connected to a third three-way tee via the coil C_2 (10 m × 0.5 mm I.D.) and mixed with the OPA/ME reagent delivered at a flow rate of 0.60 ml/min through a Mini-micro pump P_4 (Type KSU-45 H; Kyowa Seimitsu). The outlet of the coil C_3 (10 m × 0.5 mm I.D.) was connected to a 14-µl quartz flow cell in a fluorescence detector FD (Type FLD-1; Shimadzu Corp.; Kyoto, Japan) equipped with a coated low pressure mercury lamp emitting light at 300-400 nm (maximum intensity at 360 nm) and an EM-3 secondary filter which cuts off light shorter than 405 nm. The outlet of the flow cell was connected to a back pressure coil (10 m × 0.5 mm I.D.). The fluorescence intensities were recorded with a recorder R (Model EPR-100A; Toa Electronics, Tokyo, Japan).

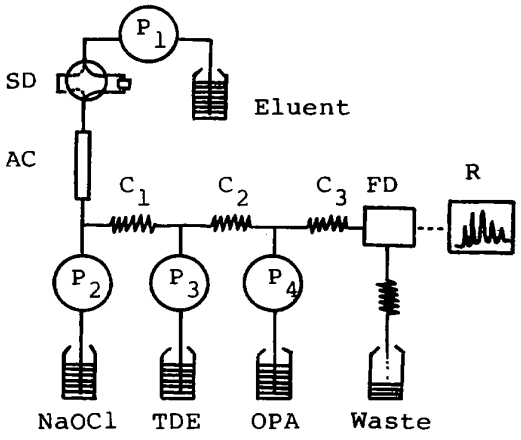


Fig. 1 Flow diagram of the HPLC system
SD: sampling device
AC: analytical column

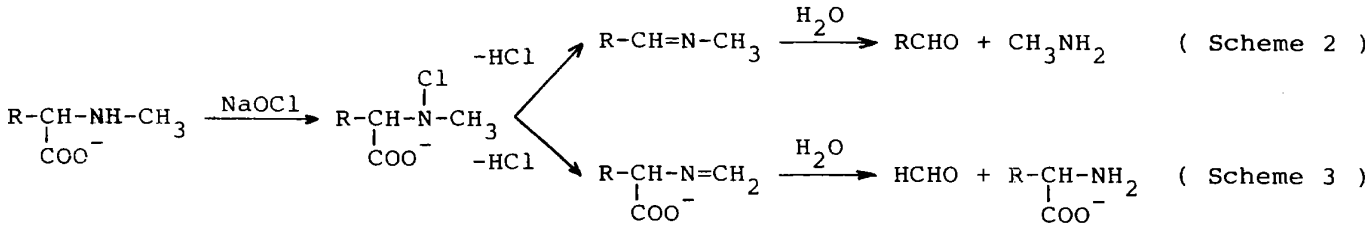
Detection of primary amines in the reaction mixtures by HPTLC

A 3-µl aliquot of the reaction mixture was spotted onto a silica gel 60 HPTLC plate, air-dried and developed with 1-butanol-acetic acid-water (5:2:3) or 1-butanol-acetic acid-water (4:1:5, upper phase) at ambient temperature. After brief drying, the plate was sprayed with 1 M carbonate buffer (pH 10.2) and then the OPA/ME reagent and the fluorescence was observed with a long-wave ultraviolet lamp in the dark.

RESULTS AND DISCUSSION

At first, we conducted the analyses of the reaction mixtures of N-methylamino acids and NaOCl. By analogy with the reaction of proline with N-chlorosuccinimide (Scheme 1), methylamine was supposed to be produced as a final nitrogenous product

from N-methylamino acids according to Scheme 2, though there remained the possibility of another pathway through which, for example, parent amino acids were produced as shown in Scheme 3. In the case of sarcosine (N-methylglycine), therefore, methy-



amine and/or glycine was considered as nitrogenous product(s). Figure 2 shows the chromatograms of the reaction mixture of sarcosine and NaOCl and of authentic compounds, obtained with a column of strong cation exchanger (Partisil-10 SCX) and 0.1 M citrate buffer (pH 2.8). Figure 2a revealed that only the peak with the same retention time as that of authentic methylamine newly appeared together with that of unreacted sarcosine, which indicated that methylamine was exclusively produced from sarosine by the reaction with NaOCl. The small peak eluted at about 7 min always appeared. Similarly, the

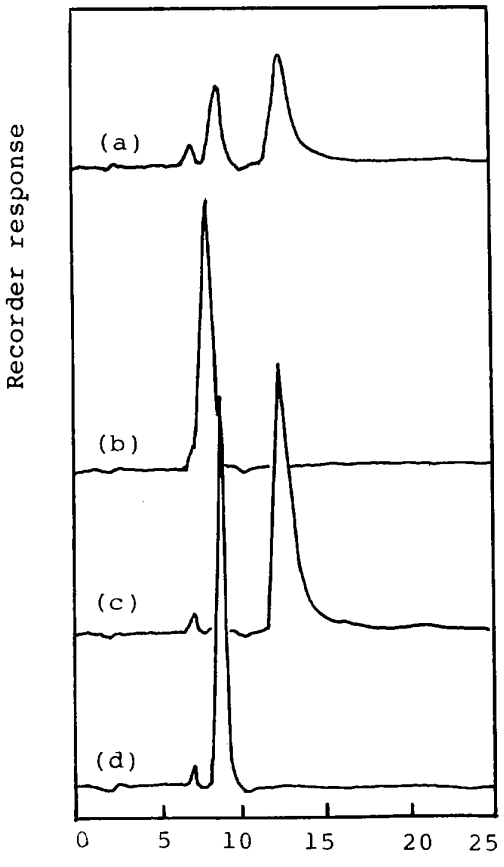


Fig. 2 Chromatograms of the reaction mixture of sarcosine and NaOCl(a) and of authentic glycine(b), methylamine (c) and sarcosine(d)

Injected amount of each authentic amine was 2.4 nmol.

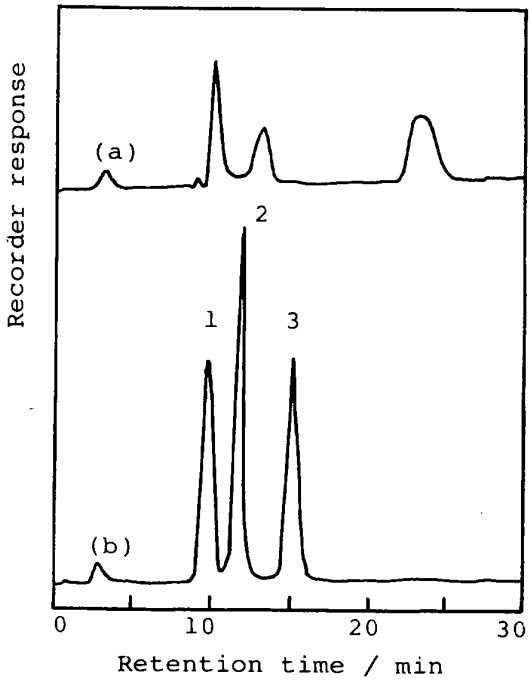


Fig. 3 Chromatograms of the reaction mixture of L-epinephrine and NaOCl(a) and of authentic compounds(b)

Peak identity: 1, methylamine(0.9 nmol); 2, L-norepinephrine(9.1 nmol); 3, L-epinephrine(3.6 nmol).

reaction mixtures of other N-methylamino acids, L-epinephrine and DL-metanephrine with NaOCl were analyzed with the Partisil-10 SCX column. All these secondary amino compounds gave new peaks which had the same retention times as that of authentic methylamine. The reversed phase TSK LS-410 column and 0.1 M citrate buffer (pH 3.0)-methanol (100:10) were used for the analyses of the reaction mixtures of L-epinephrine, DL-metanephrine, N-monosubstituted benzylamines and N-alkylanilines. Figure 3 shows the typical chromatograms obtained with L-epinephrine. Under the reaction conditions employed, the peak of L-epinephrine almost disappeared and the peak which was supposed to correspond to methylamine was observed together with unidentified peaks eluted at 13 min and 23 min. The unknown compounds exhibited native fluorescence and were thought to be oxidation products of L-epinephrine. Similarly, two uncharacterized peaks which had almost the same retention times and elution patterns as those obtained with L-epinephrine were observed in the chromatogram of the metanephrine treated with NaOCl. As shown in Fig.4, the main nitrogenous oxidation product of N-ethylbenzylamine was estimated to be ethylamine. Figure 5 depicts the result obtained with another type of secondary amine, N-methylaniline, indicating the production of methylamine but not aniline from the secondary amine. Results of these experiments are summarized in Table 1 in which the primary amines tentatively identified as nitrogenous products and their yields from secondary amines, calculated by comparing the peak areas of products with those of authentic

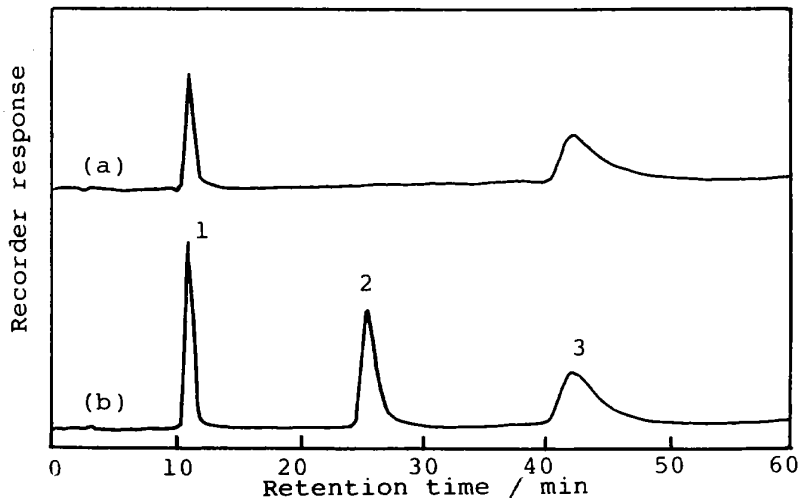


Fig. 4 Chromatograms of the reaction mixture of N-ethylbenzylamine and NaOCl(a) and of authentic compounds(b)

Peak identity: 1, ethylamine(1.1 nmol)
2, benzylamine(4.3 nmol)
3, N-ethylbenzylamine(2.1 nmol)

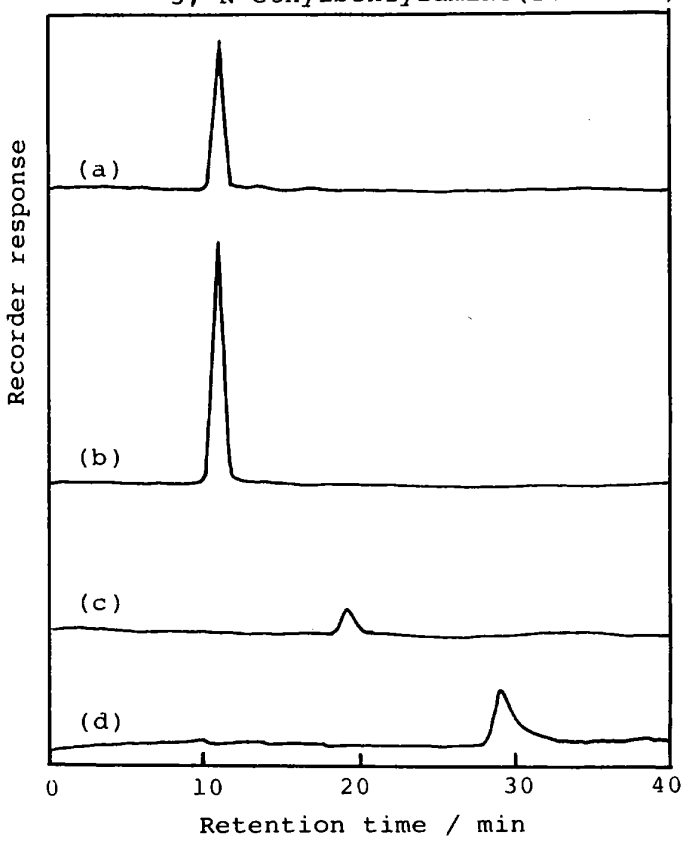


Fig. 5 Chromatograms of the reaction mixture of N-methylaniline and NaOCl(a) and of authentic methylamine(b, 1.1 nmol), aniline(c, 4.5 nmol) and N-methylaniline (d, 2.1 nmol)

Table 1 Yields and R_f values of primary amines produced in the conversion reaction

Compound	Primary amine tentatively identified by HPLC	Yield (%)	R_f value*			
			Sample		Authentic	
			A	B	A	B
Sarcosine	Methylamine	45	0.32	0.17		
N-Methyl-L-leucine	Methylamine	48	0.32	0.18		
L-Epinephrine	Methylamine	22	0.32	0.18		
DL-Metanephrine	Methylamine	40	0.32	0.18		
N-Methylbenzylamine	Methylamine	48	0.32	0.18		
N-Ethylbenzylamine	Ethylamine	27	0.43	0.24		
N-Phenylbenzylamine	Aniline	26	0.85	0.92		
	Benzylamine	2	ND**	ND		
N-Methylaniline	Methylamine	28	0.32	0.18		
N-Ethylaniline	Ethylamine	34	0.44	0.24		
N-Methylcyclohexylamine	Methylamine	7	0.32	0.19		
	Cyclohexylamine	trace	ND	ND		
Methylamine					0.32	0.18
Ethylamine					0.44	0.24
Aniline					0.85	0.92
Benzylamine					0.72	0.55
Cyclohexylamine					0.72	0.53

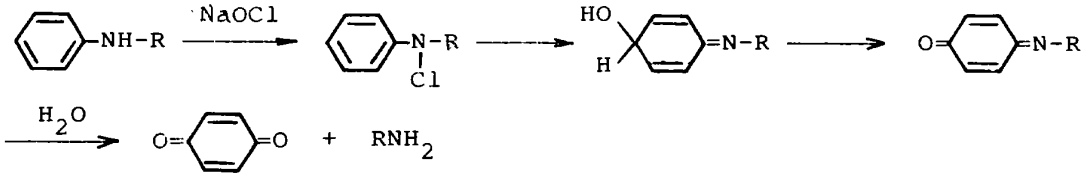
* Means of duplicate runs. A: 1-Butanol-acetic acid-water (5:2:3)
B: 1-Butanol-acetic acid-water (4:1:5, upper phase)
** ND: Not detected.

primary amines, are listed. The identities of the products were further confirmed by HPTLC as shown in Table 1. Thus, N-methylamino acids, L-epinephrine and DL-metanephrine were found to produce methylamine as the main nitrogenous product. Consequently, the reaction of N-methylamino acids with NaOCl seems to occur accoding to Scheme 2 rather than Scheme 3. Similar mechanisms are reasonably considered with cases of the secondary catecholamine and its 3-O-methyl derivative. When treated with NaOCl, N-methylbenzylamine, N-ethylbenzylamine and N-phenylbenzylamine produced methylamine, ethylamine and aniline, respectively, as a main nitrogenous product. The reaction mechanism for these N-monosubstituted benzylamines is essentially the same as for N-methylamino acids (Scheme 2) and may be formulated as shown in Scheme 4.

$$\underset{(\ R=CH_3,\ C_2H_5,\ C_6H_5\ )}{Ph\text{-}CH_2\text{-}NH\text{-}R} \xrightarrow{NaOCl} Ph\text{-}CH_2\text{-}\underset{\displaystyle Cl}{\underset{|}{N}}\text{-}R \xrightarrow{-HCl} Ph\text{-}CH{=}N\text{-}R \xrightarrow{H_2O} Ph\text{-}CHO + RNH_2 \quad (\text{Scheme 4})$$

In the previous paper[10], we assumed the formation of aniline from N-methylaniline in the reaction with NaOCl. However, as shown in Table 1, N-methylaniline actually gave methylamine rather than aniline, and N-ethylaniline gave ethylamine. A probable mechanism for the formation of alkylamines from the N-alkylanilines is proposed as illustrated in Scheme 5. The production of a minute amount of benzylamine from N-phenylbenzylamine may be understood by the partial reaction of the secondary amine with NaOCl as an N-alkylaniline according to Scheme 5.

Some secondary amines produced plural primary amines after the NaOCl treatment. For example, N-phenylbenzylamine gave aniline (26 %) and benzylamine (2 %), N-methyl-



(Scheme 5)

cyclohexylamine gave methylamine (7 %) and a trace amount of cyclohexylamine, and N-methylbutylamine gave methylamine and n-butylamine in a molar ratio of about 1:1. In these cases, the relative yields of the primary amines produced seem to be primarily determined by the difference in the acidities of protons on the carbon atoms adjacent to the chlorinated nitrogen atom. The elimination of hydrogen chloride predominantly takes place between the active chlorine atom and the neighboring proton with lower pK_a value unless the latter is sterically hindered. Usually, the acidities of methyl protons and methylene protons in aliphatic compounds are not much different from each other, while the methylene protons in the benzyl group are more acidic than the aliphatic methyl and methylene protons because of the electron-attracting property of the phenyl group. These explain why N-methylbutylamine gave equimolar amounts of methylamine and n-butylamine and why N-alkylbenzylamines predominantly produced n-alkylamines upon reacting with NaOCl.

As expected from the behavior of N-methylamino acids, the reaction mixtures of α-amino acids and NaOCl were found to contain ammonia. The percent conversion of α-amino acids to ammonia under the conditions described in the Experimental was 49 % for DL-alanine and 41 % for L-aspartic acid. The reason for the decreased sensitivities of α-amino acids in the amino acid analyses with postcolumn derivatization with NaOCl-OPA/ME has not been clarified so far. The conversion of α-amino acids to ammonia as observed in this work is considered to be the main reason for it since the fluorescence intensity of ammonia is much lower than those of α-amino acids in the OPA/ME fluorogenic reaction. The mechanism of the conversion of α-amino acids to ammonia is essentially the same as that of the Strecker degradation[13]. The susceptibility of amino acids to NaOCl or other oxidizing agents seems to be limited to α-amino acids. The existence of α-carboxyl group is obviously responsible for the enhanced elimination of hydrogen chloride through a conserted decarboxylation. In fact, other classes of amino acids such as ß-alanine and γ-aminobutyric acid or alkylamines were relatively resistant to NaOCl. Concerning the evaluation of the influence of the mode of addition of NaOCl to the eluate on the sensitivity of amino acids, there is some discrepancy between researchers who have studied the postcolumn NaOCl-OPA/ME derivatization technique. Böhlen and Mellet[3] added NaOCl to the column eluate only when proline was eluted (switching flow method) because continuous addition of NaOCl resulted in a marked reduction in the sensitivity of α-amino acids, i.e., to less than 10 %. In contrast, Ishida et al.[4] developed the " non-switching flow method " in which NaOCl is always added to the column eluate. The latter authors claimed that the addition of NaOCl had little or no influence on the sensitivities of α-amino acids and that a few picomoles of α-amino acids could be determined. However, our previous[10)11] and the present results suggest that as far as the sensitivity is concerned the switching flow method is more recommendable for the determination of α-amino acids than the non-switching flow method.

The validity of the reaction mechanisms proposed in this work requires the identification of aldehydes as another reaction products. Our attempts to detect aldehydes in the reaction mixtures of amines and NaOCl by use of acetylacetone and ammonium chloride as postcolumn reagents (Hantzsch reaction[14]) have been unsuccessful. However, we could detect carboxylic acids in the reaction mixtures obtained from such compounds as sarcosine, N-methyl-L-leucine and DL-alanine by the hydroxamate-iron (III) method[15]. If the acidic product was assumed to be formic acid, the yield of the carboxylic acid from sarcosine was 40 %. These observations suggest the occurrence of oxidation of aldehydes to the corresponding carboxylic acids during the conversion reaction, which supports the reaction scheme we presented here.

The findings obtained in the present investigation will be useful for the fluorometric determination of primary and secondary amino compounds with postcolumn NaOCl-OPA/ME derivatization.

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Keyword phrases

fluorometric detection of amines by high performance liquid chromatography; conversion of secondary amines to primary amines by sodium hypochlorite; postcolumn derivatization of primary amines with o-phthalaldehyde/2-mercaptoethanol; degradation of α-amino acids to ammonia by sodium hypochlorite; fluorescence detection of primary amines by high performance thin-layer chromatography.

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