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STUDIES ON THE IDENTITY OF THE UNKNOWN IN PROTEIN HYDROLYSATES

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SUMMARY

One of the unknown compounds frequently observed in the protein hydrolysates has been identified as methylamine. This identification is based on chromatography on the automatic amino acid analyzer and cocrystallization with authentic methylamine This artifact is most probably produced during alkaline or acid hydrolysis of protein whose arginine residues are methylated to form N^G-methylarginines. Under routine acid hydrolysis of protein such as in 6 N HCl *in vacuo* at 110°C for 24 hours, at least 10% of N^G-monomethyl-L-arginine is degraded to form methylamine.

In recent years, several investigators have reported the presence of unidentified compound(s) on the amino acid analysis of protein hydrolysates (1-8). One of these compounds was invariably eluted after ammonia, suggesting a highly basic nature of the compound. We have identified this hither-to-unknown compound as methylamine, which is the hydrolysis product of N^G-methylarginines arising from the enzymatic methylation of proteins *in vivo* and *in vitro* (9-11).

MATERIALS AND METHODS

Materials Methylamine hydrochloride, Dowex #1 resin, picric acid, L-arginine and L-ornithine were obtained from Fisher Chemical Co. and N-methylthiourea and methyl iodide from Aldrich Chemical Corp., Milwaukee, WI. [^{14}C]Methylamine.HCl (specific activity, 40 mC$_i$/mmol in ethanol), S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 50 mC$_i$/mmol in H_2SO_4) and [methyl-^{14}C]methionine (specific activity, 50 mC$_i$/mmol) were purchased from New England Nuclear, Boston, MASS. All the other reagents were obtained from various commercial sources and were of the highest grade available.

Synthesis of N^G-mono[methyl-^{14}C]-L-arginine The methyl-labeled N^G-mono[methyl-^{14}C]-L-arginine was prepared in a two-step synthesis (12). Briefly stated, [methyl-^{14}C]-amine.HCl was treated with carbon disulfide in NaOH solution at 0-5°C, and the [methyl-^{14}C]isothiocyanate thus formed was separated by treating the reaction mixture with ethyl chloroformate (13). The freshly prepared [methyl-^{14}C]isothiocyanate was then treated with concentrated NH_4OH under reflux, the solution was heated on a boiling water bath for 30 minutes, and the crystallized N-[methyl-^{14}C]thiourea was coupled with α-amino conjugated L-ornithine via N, S-dimethylthiopseudouronium iodide to synthesize N^G-mono[methyl-^{14}C]-L-arginine (12,14). The final product was more than 95% pure, contaminated only slightly with ornithine and methylurea. Specific radioactivity of N^G-mono[methyl-^{14}C]-L-arginine was 40,500 cpm/μmole of the compound (12). For the synthesis of non-labeled N^G-monomethyl-L-arginine, commercially obtainable N-methylthiourea was employed.

Amino acid analysis on automatic amino acid analyzer Proteins were routinely hydrolyzed in 6 N HCl at 110°C *in vacuo* for 24 hours or 48 hours, and the HCl was removed

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by repeated distillation under reduced pressure by means of a flash evaporator. The amino acid analysis was carried out by a Perkin-Elmer automatic amino acid analyzer with a column (0.9 x 30 cm) of Bio-Rad Aminex A-5 by the elution with 0.42 N (Na^+) sodium citrate buffer, pH 6.48, at 26°C at a flow rate of 30 ml/hour. Under these conditions, all of the methylated lysine and arginine derivatives were clearly separated from each other. When studying radioactivity profile, the radioactivity was monitored by a flow cell of a Packard Tri-Carb Model 2002 liquid scintillation spectrometer with a counting efficiency of approximately 60%.

Methylation of proteins with S-adenosyl-L-[methyl-^{14}C]methionine in vitro or [methyl-^{14}C]methionine in vivo Two species of proteins were labeled with [methyl-^{14}C], one of which was isolated rat liver nuclei methylated in vitro with S-adenosyl-L-[methyl-^{14}C]methionine and the other Neurospora crassa grown in the presence of [methyl-^{14}C]-methionine. Isolated rat liver nuclei were methylated with S-adenosyl-L-[methyl-^{14}C]methionine and H_2SO_4-insoluble protein was isolated according to the method previously described (15). It was found previously that the acid-hydrolysate of this protein contained mostly N^G-methylarginines as well as several unidentified compounds (see also top panel of Fig. 1).

As a second example of the occurrence of the unidentified compound(s) in the protein hydrolysate, Neurospora crassa was grown in 500 ml capacity Erlenmyer flask with 250 ml of Vogel's medium supplemented with 5 g of sucrose and 500 μC_i of [methyl-^{14}C]methionine (obtained from New England Nuclear). The organism was grown at 30°C for 65 hours, and was harvested by filtration and washed a few times with water. The harvested N. crassa was then treated to remove nucleic acids and phospholipids by the method described previously (16). Finally, the residual protein was hydrolyzed in 6 N HCl for amino acid analysis.

Synthesis of methylamine picrate The acid-hydrolysate of the in vitro [methyl-^{14}C]-labeled rat liver nuclear protein was charged on the automatic amino acid analyzer [a column (0.9 x 30 cm) of Bio-Rad Aminex A-5], and the column was eluted without developing ninhydrin color. The radioactivity was monitored, and the radioactive fractions corresponding to the unknown compound No. 1 in Fig. 1 were pooled, and the sample was concentrated and desalted according to the method described (17).

A portion of the desalted material (approximately 16,000 cpm) was mixed with 1 g of non-labeled methylamine hydrochloride, and methylamine picrate was synthesized according to the method described by McElvain (18). Briefly stated, the above concentrate was dissolved in about 100 ml of hot absolute alcohol, undissolved material was filtered off, and the flitrate was concentrated to about 30 ml under reduced pressure. Approximately 20 ml of saturated picric acid was then added to the above sample. Crystal appeared on standing in ice. The compound was recrystallized from hot absolute alcohol.

Picric acid is extremely quenchable substance for radioactivity scintillation counting. Thus, this was removed prior to the radioactivity counting by the following procedure. Two hundred mg of methylamine picrate weighed accurately was dissolved in 4.0 ml of hot water at about 60°C, and 2 g of Dowex No. 1 resin (Cl^- form, dried gently on suction) was added. The mixture was occasionally shaken during the period of 30 minutes, the resin was filtered off, and a portion of the filtrate was counted for radioactivity in a scintillation mixture (Formula 963, New England Nuclear Corp.) by Packard Tri-Carb liquid scintillation spectrometer with counting efficiency of approximately 85%.

RESULTS AND DISCUSSION

Occurrence of the unknown compounds in protein hydrolysates Fig. 1 illustrates the occurrence of a few uncharacterized compounds in the acid-hydrolysates of H_2SO_4-insoluble protein of isolated rat liver nuclei methylated in vitro with S-adenosyl-L-[methyl-^{14}C]methionine (upper panel) and of N. crassa protein methylated in vivo

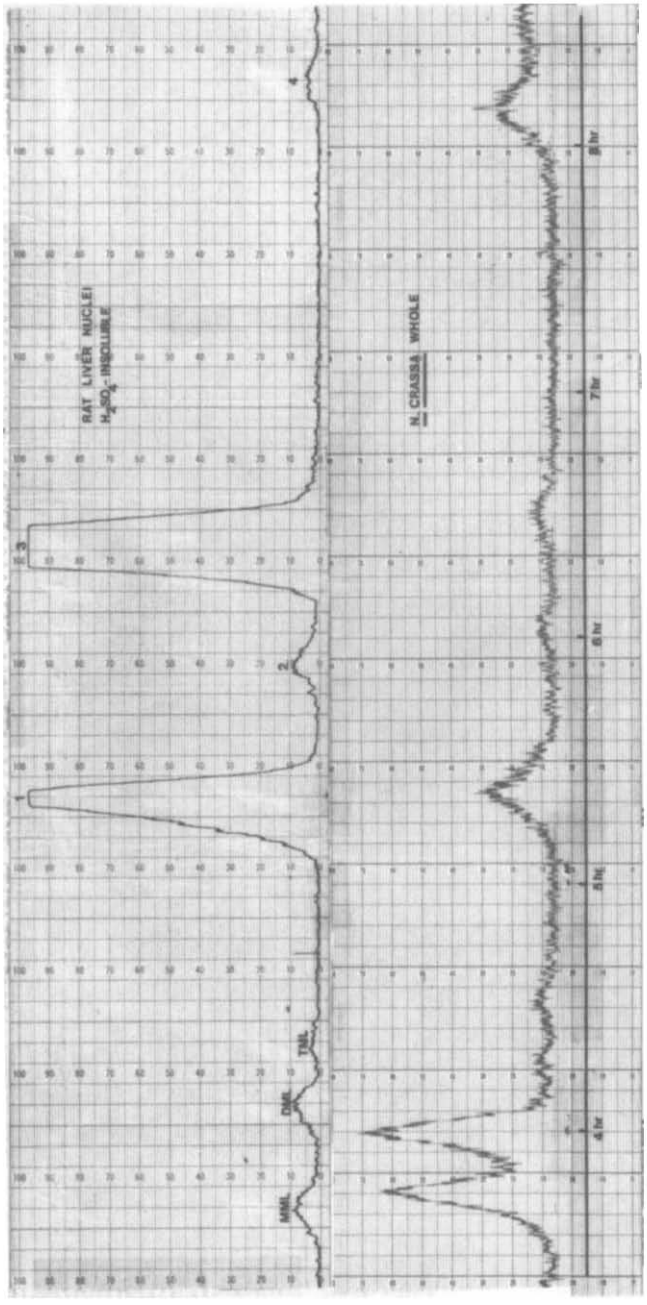

Fig. 1. Radiograms of acid hydrolysates of proteins. Top panel; Acid-insoluble protein isolated from rat liver nuclei methylated in vitro. Low panel; N. crassa protein methylated in vivo. mml, dml, tml represents ε-N-monomethyllysine, ε-N-dimethyllysine and ε-N-trimethyllysine, respectively. Detailed conditions for methylation and isolation of the proteins, as well as the acid-hydrolysis, are described under Methods.

with [methyl-^{14}C]methionine (lower panel). The unknown compound No. 1 is present in both hydrolysates. Under the elution conditions, ammonia elutes at about 4 hours and 35 minutes.

Identification of the unknown compound No. 1 with methylamine In order to study the identity of the unknown compound No. 1 in Fig. 1, the radioactive fractions corresponding to the unknown were pooled by means of the automatic amino acid analyzer, the pooled sample was mixed with non-labeled methylamine, and the mixture was again analyzed on the automatic amino acid analyzer with radioactivity monitoring. As shown in Fig. 2, the ninhydrin peak of methylamine exactly coincided with the radioactivity peak derived from the hydrolysate of the rat liver nuclear protein. The difference in the appearance between the absorption peak and the radioactivity peak of methylamine is due to a time lag of 28 minutes between the radioactivity scanner and the development of the ninhydrin color.

The identity of the above radio-labeled compound with methylamine was further established by converting the compound to its picric acid derivative. When approximately 16,000 cpm of the compound eluted from the amino acid analyzer was mixed with 1 g of non-labeled methylamine and methylamine picrate was synthesized, the following specific radioactivity was found on repeated recrystallization: 527 DPM/200 mg of methylamine picrate on 1st crystallization, 522 DPM/200 mg on the 2nd recrystallization, and 516 DPM/200 mg on the 3rd recrystallization. This constancy in the specific radioactivity of methylamine picrate on the repeated recrystallization together with the results in Fig. 2 strongly suggest that the radioactive unknown compound in the protein hydrolysate is indeed methylamine.

Alkali treatment of N^G-monomethyl-L-arginine Having established the identity of one of the unknown compounds in protein hydrolysates with methylamine, we have investigated the origin of methylamine. It is well known that arginine and its derivatives are extremely sensitive to alkali treatment (19). For example, arginine on alkali treatment degrades, yielding urea, carbon dioxide, ammonia, ornithine, and citrulline. Therefore, N^G-monomethyl-L-arginine (lower panel in Fig. 3) was treated in 1.0 ml of 0.2 N NaOH for 2 hours at 100°C, and the sample was analyzed on the amino acid analy-

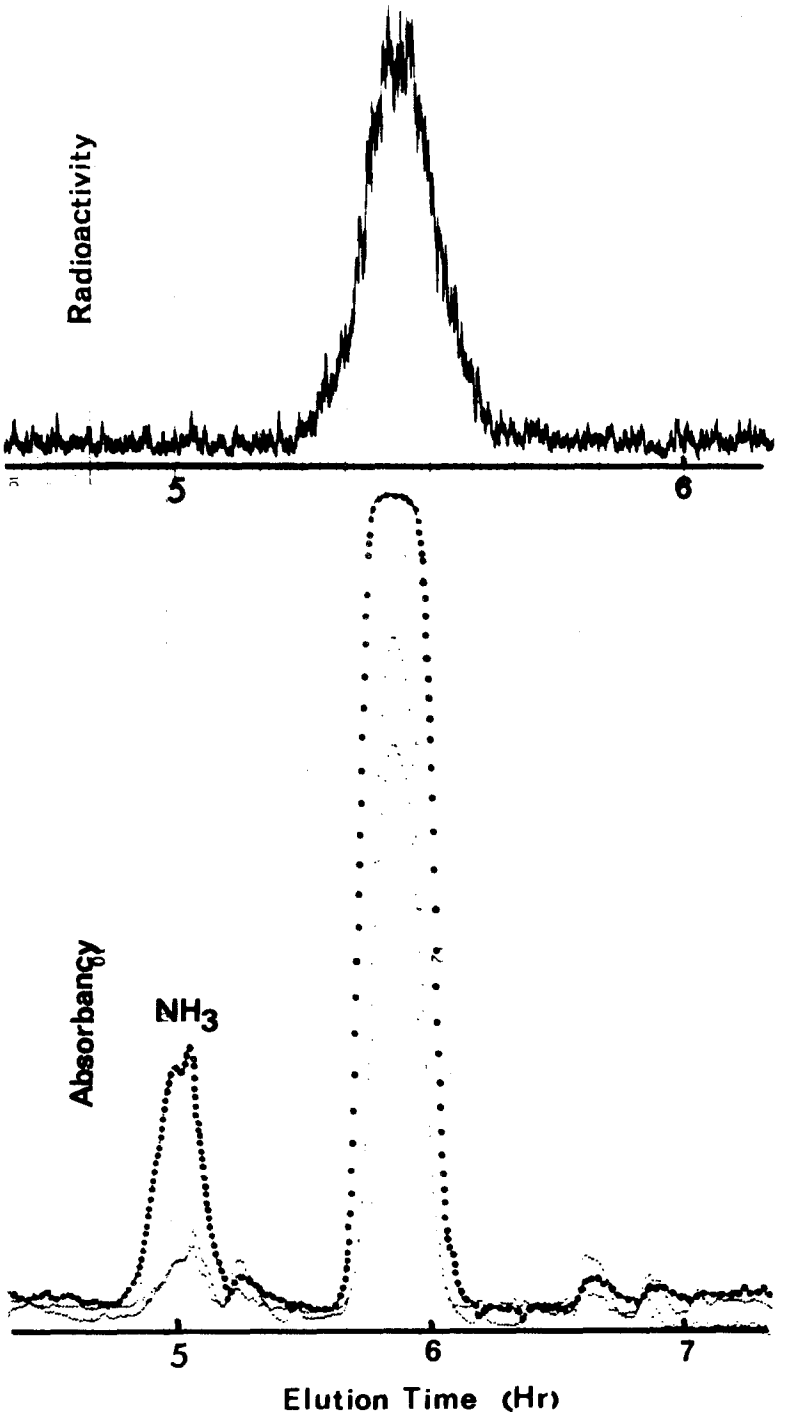
Radioactivity
5
6
Absorbancy
NH3
5
6
7
Elution Time (Hr)

Table I

Stability of N^G-mono[methyl-^{14}C]-L-arginine on acid or alkali-treatment

Treatment	Total radioactivity recovered on amino acid analyzer		% distribution of recovered radioactivity in various comp.		
	cpm	%	MU*	Methylamine	MMA*
None	23,742	95.0**	0	0	100.0
6 N HCl for 24 hrs.	21,284	85.1	2.2	1.2	96.6
0.2 N NaOH for 2 hrs & 6 N HCl for 24 hrs	5,838	23.4	12.0	79.5	8.5
0.2 N NaOH for 2 hrs	7,645	30.6	11.0	79.6	9.3

* MU and MMA represents methylurea and N^G-mono[methyl-^{14}C]-L-arginine, respectively.

** One hundred per cent represents 25,000 cpm.

ser. It is seen in the upper panel of Fig. 3 that methylurea, ornithine and ammonia were formed at the expense of N^G-monomethyl-L-arginine. In addition, there appeared an unidentified compound after ammonia. After repeating similar procedure with N^G-mono[methyl-^{14}C]-L-arginine, pooling radioactive material corresponding to the unknown, and having analyzed on the analyzer, an identical result to that in Fig. 2 was observed, proving that the unknown compound originating from the alkali treatment of N^G-monomethyl-L-arginine was methylamine.

Formation of methylamine from N^G-mono[methyl-^{14}C]-L-arginine by acid treatment As described above as well as in Table I, alkali treatment of N^G-monomethyl-L-arginine gave rise to the formation of methylurea and methylamine; approximately 80% of the recovered radioactivity was found in methylamine. However, the total radioactivity recovery after alkali-treatment is only 23-30%, indicating that a large portion of [methyl-^{14}C]amine was lost due to its volatility. Contrary to alkali-treatment, although the radioactivity recovery after acid-treatment is quite high, the treatment of N^G-mono[methyl-^{14}C]-L-arginine with 6 N HCl for 24 hours (usual condition for protein hydrolysis) yielded small but definite amount of [methyl-^{14}C]amine. This proves that methylamine could be generated from protein-bound N^G-monomethylarginine on acid-hydrolysis. Since the difference between

Fig. 2. Analysis of the identification of the unknown compound No. 1 with methylamine by the automatic amino acid analyzer with flow cell monitor The top is the radioactivity and the lower panel represents the absorbancy of the ninhydrin color. Ten μmoles of standard methylamine was added. The difference in the appearance between the radioactivity peak and the absorbancy peak of methylamine is due to a time lag of 28 minutes between the radioactivity scanner and the development of the ninhydrin color. Detailed experimental procedures are described under Methods and in the text.

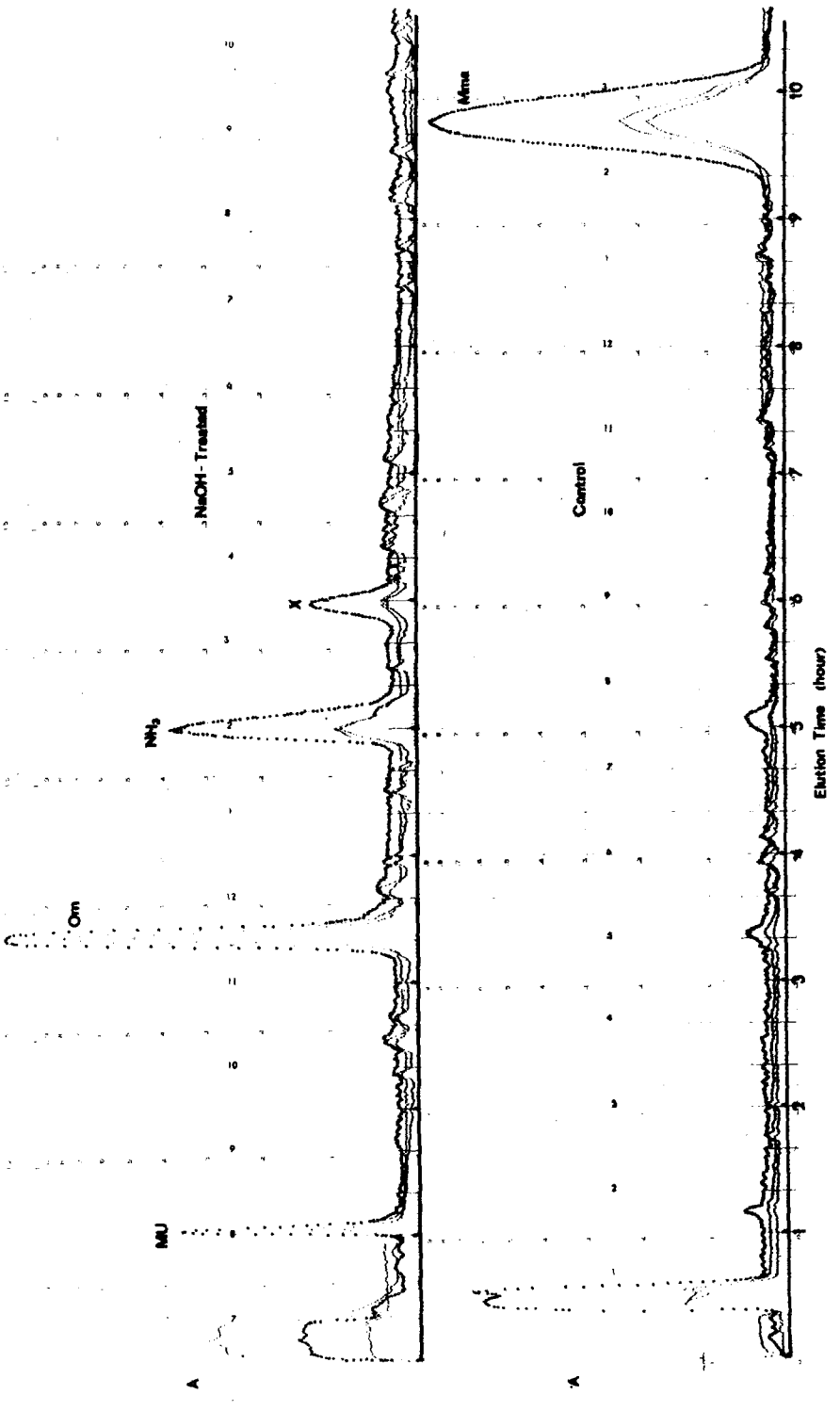

Fig. 3. Analysis of alkali-hydrolysis products of N^G-monomethyl-L-arginine. N^G-Monomethyl-L-arginine was treated in 1.0 ml of 0.2 N NaOH for 2 hours at 100°C and a portion of the sample was analyzed on the amino acid analyzer. Top panel: Alkali-treated. Low panel: Non-treated control sample. MU, Orn., Mma represents methylurea, ornithine and monomethylarginine, respectively. Detailed experimental procedures are described under Methods.

the total and methylamine recoveries of control and acid-treatment are about 10% and 1.2% respectively, it appears that acid-treatment of N^G-mono[methyl-^{14}C]-L-arginine under the present conditions degraded at least 10% of the compound originally present.

In conclusion, we have demonstrated in this paper by two independent methods that one of the hither-to-unidentified compounds in protein hydrolysates is methylamine. This identification is based on cochromatography on the amino acid analyzer and cocrystallization with standard methylamine. Methylamine is most likely produced during hydrolysis from N^G-monomethylarginine and possibly N^G-dimethylarginine which are formed posttranslationally at the polypeptide level by protein methylase I [S-adenosylmethionine:protein-arginine N-methyltransferase; EC 2.1.1.23] (20,21). N^G-Methylarginines are present ubiquitously in nature, and are rich in histone, nuclear acidic protein, encephalitogenic basic protein, myosin, HnRNP protein, ribosomal proteins and tooth matrix protein (20). Although more resistant than alkali treatment, N^G-monomethyl-L-arginine could be degraded to least 10% under routine hydrolysis condition of protein. From the foregoing considerations, therefore, it is not coincidental that the amount of this methylamine was much more pronounced whenever proteins such as HnRNP or rat liver acidic protein (7,8) were studied with [methyl-^{14}C]methionine *in vivo* or S-adenosyl-L-[methyl-^{14}C]methionine *in vitro*.

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