Note

Determination of the Yield of Norharman and Harman in the Pyrolytic Products of Proteins

Hiroshi NISHIGATA, Daisuke YOSHIDA
and Takashi MATSUMOTO*Central Research Institute, The Japan Tobacco and
Salt Public Corporation, 6-2, Umegaoka,
Midori-ku, Yokohama 227, Japan*

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We previously reported that the tryptophan pyrolyzate showed a high mutagenic activity toward *Salmonella* tester strain after metabolic activation and the co-mutagenic products norharman (I) and harman (II), were contained in the tryptophan pyrolyzate.^{1,2} These co-mutagenic activities of I and II were reported by Nagao *et al.*^{3,4} The isolation and determination of I and II from cigarette smoke were studied by Poindexter *et al.*⁵ Recently Yasuda *et al.*⁶ reported the determination of I and II in the pyrolyzates of proteins and the charred surface of roasted food by GC-Mass spectrometry. The present paper deals with the development of fluorometric analysis of them and their yields in the pyrolytic products of several proteins. In addition, the relationship between the yields of I and II in the pyrolyzate, the content of tryptophan in protein as well as the role of I and II into the mutagenicity of protein pyrolyzate are inves-

tigated.

Casein, albumin, gluten, zein and tryptophan of the purest grade available from commercial sources were used. 20 mg of each proteins or 10 mg of tryptophan were put into a small Pyrex test tube (7 mm I. D. and 0.5 mm thick). The tube was evacuated to about 10^{-3} mmHg and then sealed with a flame to a length of about 40 mm. Pyrolysis was carried out in a Packard Tri-carb Combustion Furnace Model 325. The sealed tube were inserted into the center of the pyrolysis column maintained at a predetermined temperature between 400 and 600°C and heated for 1 min. After removal and cooling, the tube was broken and its content was dissolved in methanol.

Pyrolyzate was separated into acidic, basic and neutral fractions according to the conventional liquid-liquid partition method. The basic fraction was separated by thin layer chromatography on cellulose plate (20×20 cm) using *tert*-amyl alcohol saturated with acetate buffer (pH 5.6) as the developing solvent. The spots corresponding to I and II were scraped off under UV light, and the methanol extracts were further separated by high performance liquid chromatography on a reversed-phase column, using methanol as a mobile phase. The eluates corresponding to I and II were evaporated to dryness and dissolved in 2.0 ml of dimethylsulfoxide (DMSO). Their fluorescence intensity at 290 nm of excitation spectra (emission wavelength at 380 nm) were measured by a fluorometer (Hitachi MPF-2A) and the yields of I and II were determined by narrow base line method. Calibration for narrow base line method was carried out by measuring fluorescence intensity of respective solution dissolved 10, 20, 30 and 40 ng of I or II in 2.0 ml of DMSO.

TABLE I. YIELDS OF NORHARMAN (I) AND HARMAN (II) IN THE PYROLYTIC PRODUCTS OF PROTEINS AND TRYPTOPHAN AND CONTENT OF TRYPTOPHAN IN THE PROTEINS

Protein	Yield (μ g per g protein)						Content of tryptophan in protein (%)
	I			II			
	Temperature ($^{\circ}$ C)			Temperature ($^{\circ}$ C)			
	400	500	600	400	500	600	
Zein	*	*	*	*	*	*	*
Casein	9.3 (27.8)	30.2 (90.5)	37.0 (111.0)	*	4.5 (13.4)	8.5 (25.5)	0.93
Albumin	*	16.3 (48.8)	25.9 (77.6)	*	8.6 (25.8)	12.8 (38.5)	1.02
Gluten	*	22.2 (66.6)	10.8 (32.5)	*	2.8 (8.4)	2.6 (7.9)	0.37
Tryptophan* *	4400 (13200)	19100 (57200)	35900 (107600)	1500 (4600)	16000 (48100)	17900 (53600)	100.0

* Not detected.

** μ g per g tryptophan.() μ g per g pyrolyzate.

I: R = H
II: R = CH₃

Scheme

The mutagenic activity of the pyrolytic products was evaluated according to the method of Ames *et al.*^{7,8)} The mixture of tester strain *Salmonella typhimurium* TA 98, each of properly diluted samples and the S-9 Mix was poured onto an agar over layer. After incubation at 37°C for 48 hr, histidine-revertant colonies were counted. Liver microsomal fraction (S-9) was prepared from rats that had been injected with PCB as described by Amer *et al.*^{7,8)}

The tryptophan contents in proteins were determined by Penke's method.⁹⁾

The yields of I and II in the pyrolytic products of proteins at the temperature from 400 to 600°C and the contents of tryptophan in the proteins are shown in Table I. The yield of I was higher than that of II at the same pyrolysis temperature. In smoke condensate of cigarette, the content of I was also higher than that of II.⁵⁾ Although I and II were not detected at 400°C in some of the pyrolytic products of proteins, their yields increased by elevating the pyrolysis temperature except that the yields of I and II in gluten pyrolyzate were higher at 500°C than those at 600°C. The yields of I and II in the pyrolytic product of tryptophan were over 100 times as much than those in the protein pyrolyzate.

The I and II were not detected in the pyrolytic product of zein which contained no tryptophan. The yields of I and II were lowest for gluten pyrolyzate corresponding to the lowest tryptophan content among tested proteins. These results suggested that I and II might be derived from tryptophan moiety of proteins.

The mutagenic activity of the pyrolytic products of proteins and tryptophan at the temperature from 400 to 600°C are shown in Fig. 1. The number of revertant colonies per 100 µg of protein pyrolyzate and 50 µg of tryptophan pyrolyzate increased at the pyrolysis temperature between 400 to 600°C.

In this experiment, 100 µg of pyrolyzate of proteins was added to one plate for assay and less than 1 µg of I and II were to be contained in the pyrolyzate. It has been reported that the distinct enhancing effect of II on the mutagenicity was observed at the level of 50 µg per plate.²⁾ Therefore, neither I nor II were expected to contribute to the enhancement of the mutagenicity of the pyrolyzates of proteins.

FIG. 1. Mutagenic Activities of Pyrolytic Products of Proteins and Tryptophan.

Pyrolyzates were tested to determine the mutagenic activity with the strain TA 98 in the presence of S-9 Mix. The number of His⁺ revertant colonies per 100 µg of protein pyrolyzate or 50 µg of tryptophan pyrolyzate were presented after subtraction of the average number of spontaneous revertant colonies (30). ○, casein pyrolyzate; ●, albumin pyrolyzate; △, gluten pyrolyzate; ▲, zein pyrolyzate; □, tryptophan pyrolyzate.

REFERENCES

- 1) T. Matsumoto, D. Yoshida, S. Mizusaki and H. Okamoto, *Mutation Res.*, **48**, 279 (1977).
- 2) T. Matsumoto, D. Yoshida, S. Mizusaki and H. Okamoto, *ibid.*, **56**, 85 (1977).
- 3) M. Nagao, T. Yahagi, T. Kawachi, T. Sugimura, T. Kosuge, K. Tsuji, K. Wakabayashi, S. Mizusaki and T. Matsumoto, *Proc. Jpn. Acad.*, **53**, 95 (1977).
- 4) M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Matsushima and T. Sugimura, *ibid.*, *Ser. B*, **53**, 34 (1977).
- 5) E. H. Poindexter and R. D. Carpenter, *Phytochem.*, **1**, 215 (1962).
- 6) T. Yasuda, Z. Yamaizumi, S. Nishimura, M. Nagao, Y. Takahashi, H. Fujiki, T. Sugimura and K. Tsuji, Proceedings of the Japanese Cancer Association The 37th Annual Meeting, Tokyo, August, 1978, p. 21.
- 7) B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.*, **31**, 347 (1975).
- 8) B. N. Ames, W. E. Durston, E. Yamazaki and F. D. Lee, *Proc. Natl. Acad. Sci. (U.S.A.)*, **70**, 2281 (1973).
- 9) B. Penke, R. Ferenczi and K. Kovacs, *Anal. Biochem.*, **60**, 45 (1974).