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# Isolation and Structure Studies of Mutagenic Principles in Amino Acid Pyrolysates

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The present paper describes the detail of the serach of mutagenic principles in tars from amino acids, especially from tryptophan. A series of amino acids was pyrolyzed and was tested their mutagenicity to find that basic fractions from pyrolysates of tryptophan, ornitine, glutamic acid, serine, lysine and creatine showed potent mutagenicities to TA98 than the fractions from pyrolysates of other amino acids. Among them the active principles in the pyrolysate of tryptophan were identified as 3-amino-1,4-dimethyl-5H-pyrido-[4,3-*b*]indole (designated as Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (designated as Trp-P-2).

Among several antimicrobial tar constituents, it was found that 2-amino-5-phenylpyridine, benzo[*f*]quinoline and phenanthridine also were mutagenic.

**Keywords**——mutagen; tryptophan; tar constituent; 5H-pyrido[4,3-*b*]indol; amino-carboline; carcinogen; aminopyridine; phenylalanine; benzo[*f*]quinoline; amino acid

Much attension has been paid for a long time that smoke condensate, charred material and other pyrolysis products are doubted to be carcinogenic.[2,3] Recently, Sugimura and co-workers reported that those pyrolysates showed high mutagenic activity in the Ames test.[4,5]

For the past decade ago, we have been searching biologically active components from various tars, including amino acid pyrolysates, and a number of antimicrobial and antihistamic compounds has been identified.[6] The present paper describes the details on the search of mutagenic principles in the tars from amino acids, especially from tryptophan.[7]

Mutagenic tests were carried out using an improved method[8] of the Ames test.[9] TA98 and TA100 strains of *Salmonella typhimurium* were kindly supplied by Dr. B.N. Ames, University of California.

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Table I. Mutagenic Activities of Pyrolysates of Amino Acids

| Amino acid | Fraction[a] | Yield (mg) | TA 100 Revertants/mg | | TA 98 Revertants/mg | |
|---|---|---|---|---|---|---|
| | | | +S-9 | −S-9 | +S-9 | −S-9 |
| D,L-Tryptophan (10 g) | Tar | 6500 | 2500 | 105 | 22420 | 16 |
| | Basic fract. | 1580 | 3670 | 118 | 35200 | 21 |
| | A.N. fract. | 3310 | 1670 | 101 | 250 | 19 |
| L-Ornithine-HCl (5 g) | Ether sol. | 359 | 560 | k[b] | 4990 | 13 |
| | MeOH sol. | 2359 | 1800 | k[b] | 7300 | k[b] |
| | Basic fract. | 195 | 620 | k[b] | 24950 | 39 |
| | A.N. fract. | 23 | 248 | k[b] | 165 | 40 |
| L-Glutamic acid (20 g) | Ether sol. | 764 | 3408 | 53(k)[b] | 25515 | 24 |
| | MeOH sol. | 777 | 2020 | 163 | 3200 | 47 |
| | Basic fract. | 236 | 7030 | k[b] | 22800 | 15 |
| | A.N. fract. | 216 | 295 | k[b] | 374 | k[b] |
| L-Serine (5 g) | Ether sol. | 198 | 2440 | 114 | 16783 | 8 |
| | MeOH sol. | 621 | 2600 | 137 | 20412 | 22 |
| | Basic fract. | 52 | 5640 | 128 | 19732 | 20 |
| | A.N. fract. | 10 | 492 | 161 | 461 | 31 |
| Creatinine (10 g) | Tar | 1785 | | | 5900 | 21 |
| | Basic fract. | 599 | | | 12000 | 24 |
| | A.N. fract. | 130 | | | 466 | 27 |
| Creatine (10 g) | Tar | 1785 | | | 8700 | 17 |
| | Basic fract. | 504 | | | 10000 | 32 |
| | A.N. fract. | 114 | | | 479 | 27 |
| L-Lysine-HCl (10 g) | Ether sol. | 345 | 608 | k[b] | 4240 | 27 |
| | MeOH sol. | 4440 | 2000 | k[b] | 5300 | 21 |
| | Basic fract. | 230 | 1360 | k[b] | 6544 | 7 |
| | A.N. fract. | 79 | 315 | 48 | 122 | 5 |
| L-Arginine-HCl (20 g) | Ether sol. | 1065 | 1740 | k[b] | 4763 | 3 |
| | MeOH sol. | 2514 | 1468 | 61(k)[b] | 7144 | 32 |
| | Basic fract. | 455 | 2097 | 29(k)[b] | 4309 | 17 |
| | A.N. fract. | 69 | 358 | 47(k)[b] | 107 | 22 |
| L-Citrulline (4.7 g) | Ether sol. | 206 | 1700 | 38(k)[b] | 3289 | 13 |
| | MeOH sol. | 1116 | 1900 | 110 | 4933 | 32 |
| | Basic fract. | 88 | 1436 | 40(k)[b] | 5443 | 16 |
| | A.N. fract. | 29 | 377 | 66(k)[b] | 218 | 18 |
| L-Threonine (20 g) | Ether sol. | 2733 | 1286 | k[b] | 3062 | 9 |
| | MeOH sol. | 437 | 1440 | 166 | 3175 | 30 |
| | Basic fract. | 1700 | 835 | 73 | 3600 | 18 |
| | A.N. fract. | 245 | 234 | 71 | 88 | 14 |
| D,L-Alanine-HCl (5 g) | Ether sol. | 95 | 364 | 143 | 372 | 16 |
| | MeOH sol. | 1848 | 2120 | 46 | 2160 | 12 |
| | Basic fract. | 48 | 2420 | 28 | 2800 | 20 |
| | A.N. fract. | 11 | 210 | 29 | 75 | 25 |
| L-Cystine (10 g) | Ether sol. | 1081 | 965 | 132 | 2490 | 13 |
| | MeOH sol. | 552 | 572 | 190 | 1580 | 69 |
| | Basic fract. | 427 | 1760 | 47 | 1920 | 4 |
| | A.N. fract. | 304 | 226 | 118 | 139 | 4 |
| L-Glutamine (5 g) | Ether sol. | 63 | 1420 | 105 | 1940 | 30 |
| | MeOH sol. | 330 | 518 | 133 | 1000 | 25 |
| | Basic fract. | 8 | 5235 | 125 | 8933 | 97 |
| | A.N. fract. | 5 | 527 | k[b] | 355 | k[b] |
| D,L-Methionine (10 g) | Ether sol. | 1002 | 493 | k[b] | 563 | 1 |
| | MeOH sol. | 536 | 423 | 80 | 1985 | 37 |
| | Basic fract. | 592 | 610 | k[b] | 584 | 33 |
| | A.N. fract. | 64 | 330 | 66 | 174 | 17 |

| Amino acid | Fraction | Yield (mg) | TA 100 Revertants/mg | | TA 98 Revertants/mg | |
|---|---|---|---|---|---|---|
| | | | +S-9 | −S-9 | +S-9 | −S-9 |
| L-Cysteine.HCl | Ether sol. | 97 | 271 | 135 | 34 | 15 |
| (3 g) | MeOH sol. | 1225 | 341 | 52 | 392 | 15 |
| | Basic fract. | 6 | 230 | 106 | 85 | k[b] |
| | A.N. fract. | 64 | 282 | 148 | 31 | 19 |
| L-Tyrosine | Ether sol. | 941 | 231 | 76 | 142 | 16 |
| (10 g) | MeOH sol. | 2446 | 304 | 74 | 261 | 24 |
| | Basic fract. | 29 | 2380 | 45 | 2320 | 2 |
| | A.N. fract. | 564 | 172 | 51 | 57 | 3 |
| L-Phenylalanine | Tar | 4800 | 110 | | 103 | |
| (10 g) | Basic fract. | 3200 | 110 | | 200 | |
| | A.N. fract. | 700 | 140 | | 230 | |
| L-Histidine | Ether sol. | 11 | 1320 | 115 | 4309 | 18 |
| (5 g) | MeOH sol. | 2224 | 176 | 107 | 85 | 25 |
| | Basic fract. | 12 | 536 | 184 | 1440 | 65 |
| | A.N. fract. | | | | | |
| D,L-Asparagine | Ether sol. | 25 | 400 | 140 | 713 | 64 |
| (10 g) | MeOH sol. | 343 | 111 | 101 | 32 | 32 |
| D,L-Valine-HCl | Ether sol. | 80 | 146 | 6 | 24 | 5 |
| (11 g) | MeOH sol. | 1558 | 168 | 120 | 39 | 14 |
| | Basic fract. | 20 | 199 | 67 | 68 | 17 |
| | A.N. fract. | 24 | 258 | 112 | 51 | 17 |
| Albumine | Tar | 4000 | 1260 | k[b] | 5400 | k[b] |
| (10 g) | Basic fract. | 800 | 2980 | k[b] | 11400 | k[b] |
| | A.N. fract. | 1100 | 260 | k[b] | 391 | k[b] |

*a*) Ether sol., MeOH sol., basic fract. and A.N. fract. (acidic-neutral fract.) were appeared in Chart 1.
*b*) k shows the killing effect of the fraction.

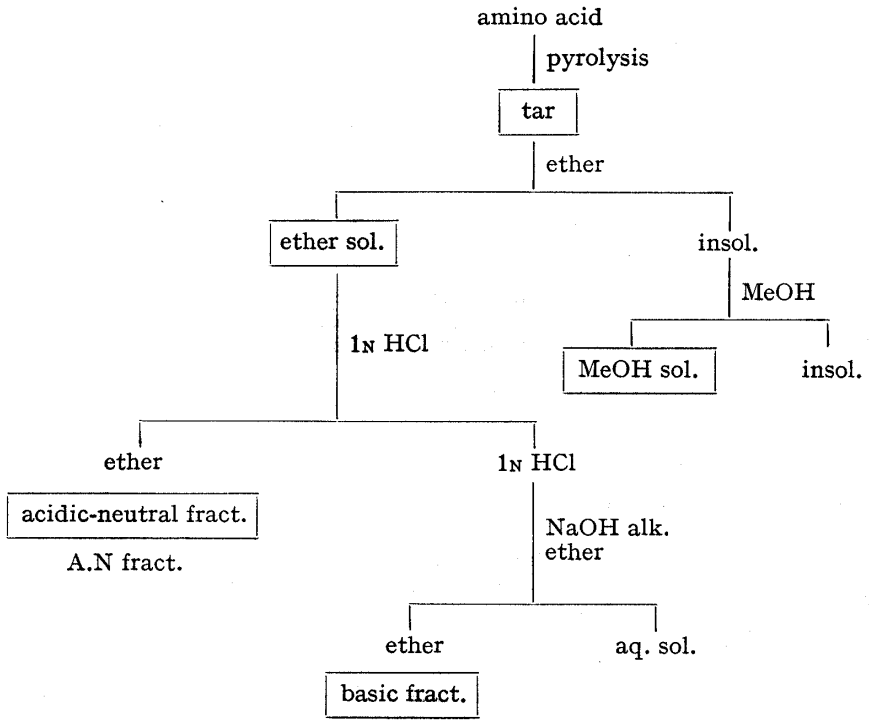
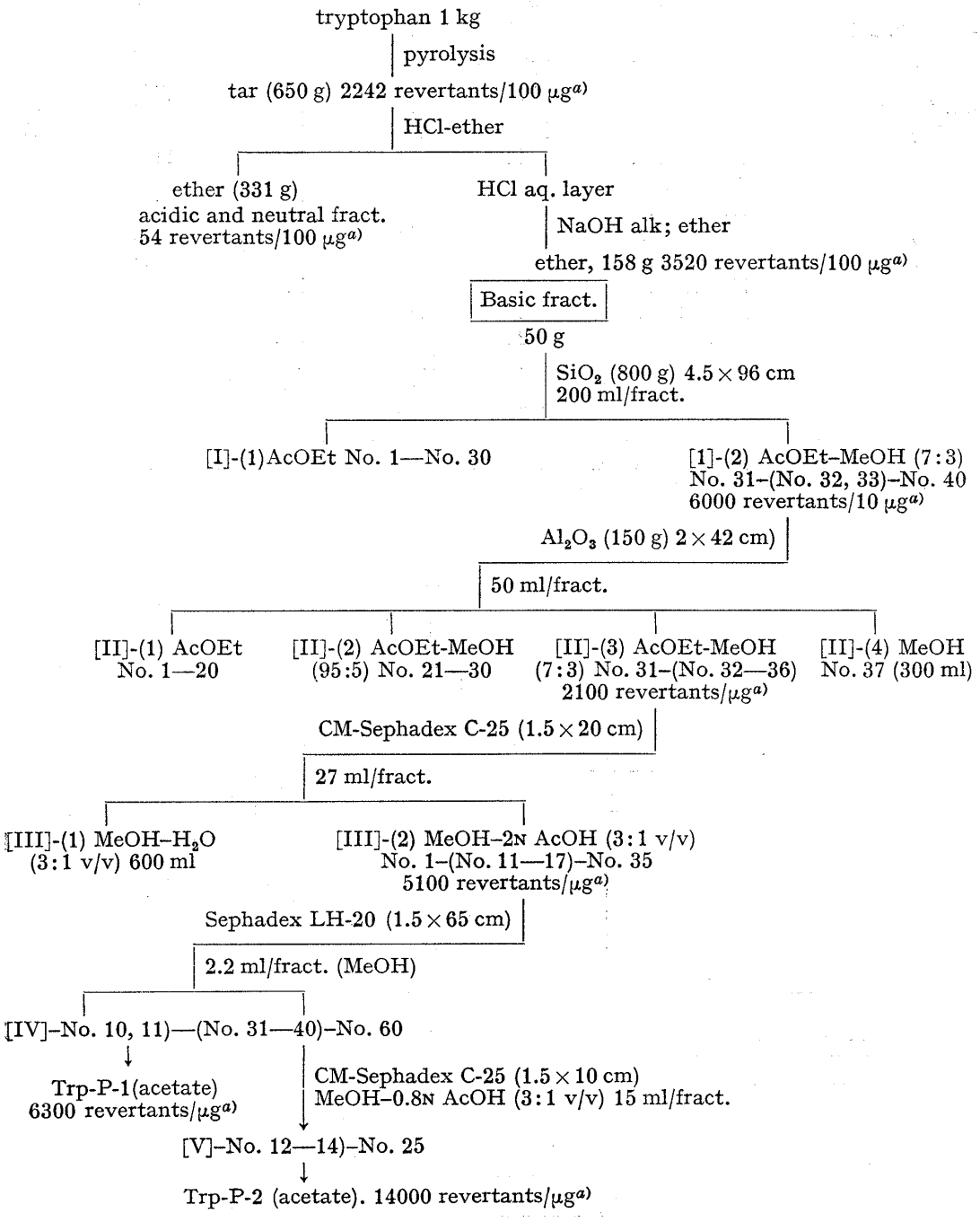



Chart 1. Separation Procedure of Acidic-neutral and Basic Fractions in Pyrolysates of Amino Acids

A series of amino acids in a flask with a thick side arm was pyrolyzed and dry-distilled by a direct flame. The distillate was separated into a basic and an acidic-neutral fraction as shown in the Chart 1. The mutagenic activity of each fraction was tested and the results were shown in the Table I. Without microsomal enzyme system (S-9 mix), each fraction did not show mutagenic activity towards both strains of TA98 and TA100. In the presence of S-9 mix, the basic fractions from most of amino acids were mutagenic but the acidic-neutral fractions were not, and TA98 was in general more sensitive than TA100. The basic fractions from pyrolysates of tryptophan, ornithine, glutamic acid, serine, lysine and creatine showed more potent mutagenicity towards TA98 than the fractions from pyrolysates of other amino




*a*) Mutagenic activity to TA98 with S-9 mix.

Chart 2. Isolation Procedure of Trp-P-1 and Trp-P-2

acids. The pyrolysate of albumin also showed mutagenicity.

Our first attempt was to identify active principles in the pyrolysate of tryptophan, which showed the highest mutagenic activity.

D,L-Tryptophan (1.0 kg) was pyrolyzed by a direct flame to give a tar (650 g). Fractionation as shown in the Chart 2 gave 158 g of a basic mixture. The mutagenicity of the fraction to TA98 was 3520 revertants/100 μg/plate. 50 g of the basic fraction was fractionated by a silica gel column using ethyl acetate and a mixture of ethyl acetate and methanol as elution solvents. Mutagenic substances were eluted in fractions No. [I]—32—33(1194 mg, 6000 revertants/10 μg/plate) (Fig. 1). These two fractions were combined and subjected to alumina column chromatography using ethyl acetate and methanol. Mutagenic compounds were recovered in fractions No.[II]—32—36(160 mg, 2100 revertants/μg/plate) (Fig. 2). Further

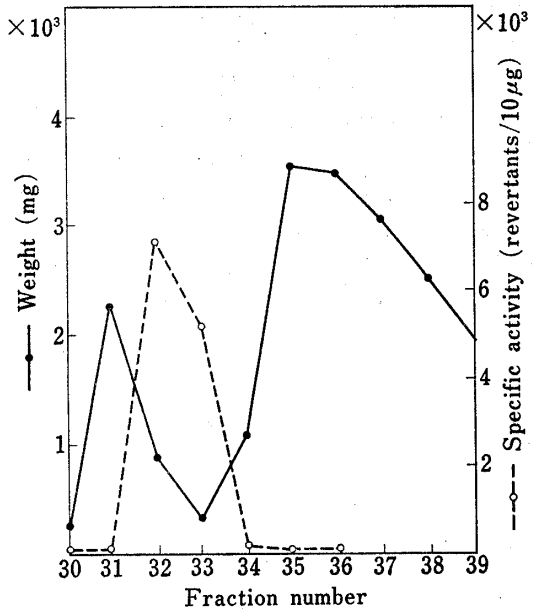



Fig. 1. Silica Gel Chromatogram of the Basic Fration

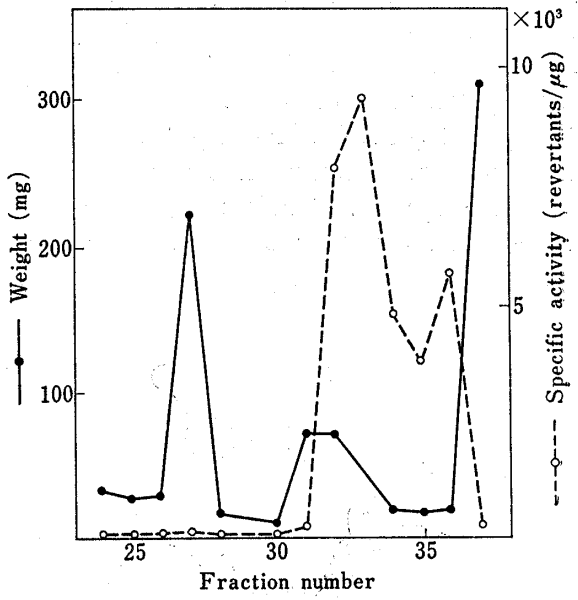



Fig. 2. Alumina Chromatogram of Fraction No. [1]—32—33

purification was carried on by converting the fraction into a water-soluble type, namely the acetate salt. Separation column was CM-Sephadex C-25, and the fractions were eluted by methanol–2N acetic acid (3: 1) to give the active fractions No.[III]—11—17. Since the elution weight and the activity curve were well coincided as shown in Fig. 3, the active principles were seemed to be purified well. More efficient separation of the active components

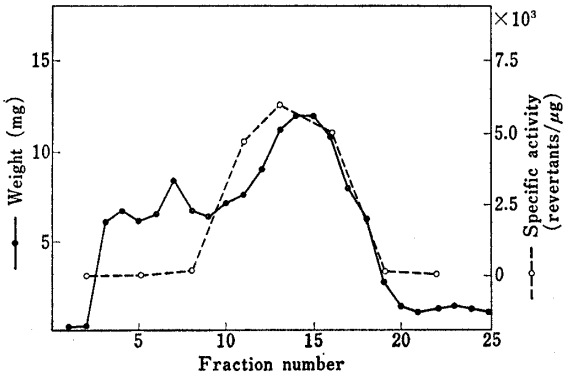



Fig. 3. CM-Sephadex C-25 Chromatogram of Fraction No. [II]—32—36

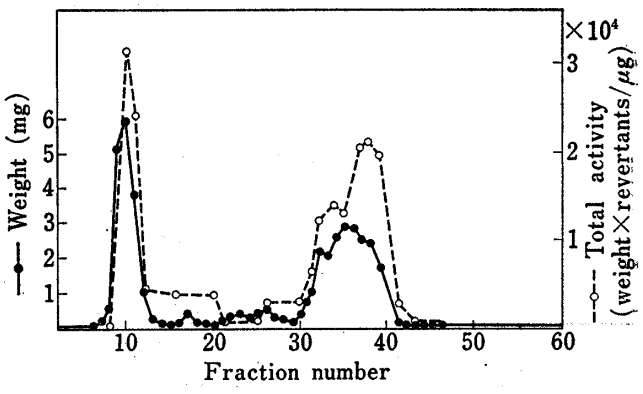



Fig. 4. Gel Filtration of Fraction No. [III]—11—17 on Sephadex LH-20

was effected by a Sephadex LH-20 column. The methanol elution gave two main peaks with mutagenic activity maxima(Fig. 4). The first peak (fraction No.[IV]—10—11), after recrystallization from acetone–methanol, gave 5 mg of a crystalline substance which was designated as Trp-P-1 acetate,[7] whose activity was 6300 revertants/μg/plate. The second active peak (fraction No. [IV]—31—40) was re-chromatographed on CM-Sephadex C-25. The fractions No. [V]—12—14, after crystallization by ethyl acetate, gave 10 mg of needles, which was designated as Trp-P-2 acetate.[7] The mutagenic activity was 14000 revertants/μg/plate.

Recovery of the activity from the crude basic fraction was about 10 percents. A later work showed that both harman and norharman which have already been identified in the basic fraction[6b,c] are co-mutagenic.[10] Considering the activity enhancement by the presence of these co-mutagens in the crude tar, the recovery of the acrivity rose up to reasonable.

Trp-P-1 acetate was recrystallized from ethyl acetate to pale brown colored needles or small prisms, mp 252—262° (the crystal form changed at about 200°). Elemental analysis showed a molecular formula of $C_{15}H_{17}O_2N_3$($C_{13}H_{13}N_3$–$CH_3COOH$). Mass spectrum suggested a molecular ion of $C_{13}H_{13}N_3$ (211.1079, calcd. 211.1082). Similarly, Trp-P-2 acetate which was recrystallized from ethyl acetate and methanol, mp 248—250°, was analyzed as $C_{14}H_{19}O_4N_3$($C_{12}H_{11}N_3$–$CH_3COOH$–$2H_2O$), and mass spectrum suggected $C_{12}H_{11}N_3$. Ultraviolet(UV) spectrum of both compounds were almost superimposable, indicating the presence of the same chromophore. Nuclear magnetic resonance spectra (NMR), though it was taken in fairly dilute solution, showed that Trp-P-1 acetate has three methyl groups, and Trp-P-2 acetate has two methyl groups, respectively, and some aromatic hydrogens.

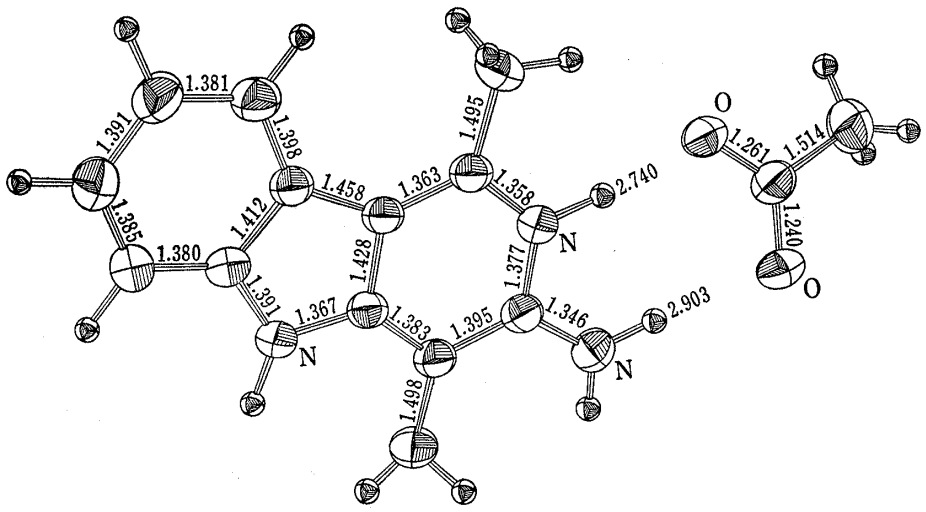
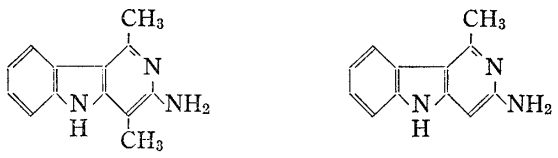



Fig. 5. A Perspective Drawing of the Acetate Salt of Trp-P-1 with C-C(or N, O) Bond Lengths (Å)




Trp-P-1 Trp-P-2

Fig. 6

10) *a*) M. Nagao, T. Yahagi, T. Kawachi, T. Sugimura, T. Kosuge, K. Tsuji, K. Wakabayashi, S. Mizusaki, and T. Matsumoto, *Proc. Japan. Acad.*, 53, 95 (1977); *b*) M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Matsushima, and T. Sugimura, *ibid.*, 53, B, 34 (1977); *c*) M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Kawachi, T. Sugimura, K. Wakabayashi, K. Tsuji, and T. Kosuge, *Cancer Letters*, 3, 339 (1977).

The structure of Trp-P-1 acetate was determined by X-ray crystallography. A crystal of size of $0.05 \times 0.1 \times 0.2$ mm, was used for X-ray experiments. It belonged to the monoclinic space group $P2_1/a$ with four molecules in a unit cell of the dimensions, $a=12.687$, $b=11.146$, $c=10.104$ Å, $\beta=103.19°$. The structure was solved by the direct method using the program MULTAN and refined by the block-diagonal least-squares method. Successive least-squares calculation led the R-index of 0.04, assuming the anisotropic thermal parameters for non-hydrogen atoms and the isotropic ones for hydrogen atoms. A perspective view of the whole molecule is illustrated in Fig. 5. The chemical structure is an acetate salt of 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Fig. 6). This is quite consistent with elemental analysis, and NMR, and UV spectra.

The structure of Trp-P-2 acetate was analyzed by NMR. The absence of the higher methyl (2.11 ppm) which is present in Trp-P-1 suggests that the remaining methyl locates

Table II. Mutagenic Activty of Compounds isolated from Various Tar Pyrolysates

| Material pyrolyzed | Compound | Revertants/100μg | | | |
|---|---|---|---|---|---|
| | | TA 100 | | TA 98 | |
| | | +S-9 | −S-9 | +S-9 | −S-9 |
| Egg yolk | Norharman | 194 | 99 | 13 | 27 |
| | Harman | 191 | 128 | 17 | 25 |
| | 1-Isobutyl-β-carboline | 157 | 131 | 46 | 21 |
| | 1-Isopentyl-β-carboline | 164 | 35 | 39 | 0 |
| | 1-(2-Methylbutyl)-β-carboline | 197 | 46 | 62 | 5 |
| | 2-Butylbenzimidazole | 103 | 89 | 19 | 8 |
| | 2-Isopentylbenzimidazole | 117 | 75 | 25 | 5 |
| | 3-Dodecylpyridine | 40/μl | 0/μl | 16/μl | 3/μl |
| | 3-Tetradecylpyridine | 109/μl | 79/μl | 16/μl | 6/μl |
| | 2-Isopropyl-7-azaindole | 130 | 107 | 17 | 17 |
| | 2-Isobutyl-7-azaindole | 176 | 124 | 48 | 22 |
| | 2-Nonyl-5,6,7,8,-Tetrahydroquinoline | 120 | 10 | 25 | 15 |
| Non-fat soybean | Norharman, Harman | | | | |
| | Phenanthridine | 399 | 73 | 75 | 3 |
| | Benzo[*f*] quinoline | 690 | 82 | 87 | 8 |
| Wood | 1,1′,3,3′-Tetraketo-2,2′-bicyclopentyl | 120 | 102 | 23 | 10 |
| | 1,1′,3,3′-Tetraketo-4-methyl-2,2′-bicyclopentyl | 127 | 92 | 21 | 3 |
| | Acetovanilone | | | | |
| Tryptophan | Norharman, harman | | | | |
| | 1-Ethyl-β-carboline | 125 | 98 | 38 | 27 |
| D,L-Tryptophan+L-leucine | Norharman, harman, 1-ethyl-β-carboline, 1-isobuthl-β-carboline, 1-isopentyl-β-carboline | | | | |
| D,L-Tryptophan+L-isoleucine | Norharman, harman, 1-ethyl-β-carboline, 1-(2-methylbutyl)-β-carboline | | | | |
| L-Phenylanine | 3-Phenylpyridine | | 190 | 30 | 27 |
| | 2-Amino-5-phenylpyridine | 2432 | 100 | 3000 | 11 |
| L-Tyrosine | 4,4′-Dihydroxybenzyl | 147 | 109 | 18 | 11 |
| | 4-Hydroxybenzyl-*p*-tolyl ether | 147 | 104 | 11 | 4 |
| | 4,4′-Dihydroxy-*trans*-stilbene | | | | |
| | N-Phenyltyramine | 204 | 81 | 31 | 9 |
| | 3-*p*-Hydroxyphenylpyridine | 148 | 77 | 35 | 14 |
| | 3-*p*-Hydroxyphenyl-6-methylquinoline | 204 | 50 | 44 | 11 |
| | 4′-Amino-2-hydroxydiphenylmethane | 155 | 112 | 23 | 22 |
| L-Lysine | 4-Azafluorene | 160 | 120 | 45 | 16 |
| | 2′,3′,4′,5′,6′,7′-Hexahydroazepino-(1′:2′-1:2)benzimidazcle | 125 | 109 | 13 | 5 |
| L-Arginine | 2-Butylbenzimidazole | | | | |
| Control | | 110—130 | 100—130 | 16—25 | 8—13 |

at the 1 position, since the methyl group at the position should be more deshielded by the benzene ring in the molecule. The appearance of a singlet absorption at 6.35 ppm is assigned to the hydrogen at $C_4$ rather than the hydrogen at $C_1$, because of its higher chemical shift. From these results and quite similar UV spectrum to that of Trp-P-1, the chemical structure of Trp-P-2 acetate was proposed as an acetate salt of 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Fig. 6). The structure was recently confirmed by a synthesis of Trp-P-2 acetate.[11]

Isolation studies of mutagenic pyrolysates of the other amino acids are in progress.

Several antimicrobial tar constituents have been isolated during the past decade.[2] Their mutagenic activities were all tested(Table II). Among them, 2-amino-5-phenylpyridine which has been isolated from D,L-phenylalanine pyrolysate[6f] was strongly mutagenic (3000 revertants /100 μg/plate in TA98). Detailed data will be published in a full paper elsewhere. And benzo[*f*]quinoline and phenanthridine isolated from non-fat soy bean tar[6c] also showed mutagenic activity, though the activity was not so strong.

The present study suggests that there are many mutagenic principles, probably very difficult to avoid, in charred foods including hydrocarbons such as benzopyrene. The precise evaluation of mutagenicity of cooked foods and whole identification of the active components are needed for the development of less-dangerous cooking methods of daily foods. The correct estimation of the degree of carcinogenicity of synthetics such as drugs, herbicides, and other enviromental substances, in comparison of power with daily cooked foods.

## Experimental

**Mutation Assay**——Assay was carried out by modifying the method of Ames as described previously,[8] namely, S-9 was prepared from the liver of SD-rat weighing 100 g pretreated with polychlorinated biphenyl. S-9 mix is a mixture of S-9 and NADH, NADPH, ATP and glucose-6-phosphate as cofactors. Test compound dissolved in 0.1 ml dimethylsulfoxide was preincubated with 0.1 ml bacterial tester strain and 0.5 ml S-9 mix for 20 min at 37°. This mixture was added 2 ml of molten soft agar and spread over minimal glucose agar plate containing 0.1 μmol of L-histidine and incubated for 2 days at 37°.

**Pyrolysis of Amino Acids**——Pyrolysis Techniques: Pyrolysis was performed over a direct gas burner flame in a Pyrex flask of enough volume with a thick side arm. Volatile product was collected in two traps cooled in ice and escaped gas was bubbled in MeOH to avoid the contamination in the air.

Fraction of Pyrolysates (Chart 1): The traps and the flask were washed with MeOH and $CHCl_3$. After evaporation of the solvents, the both residues were combined and dissolved in ether. The ether solution was extracted with 1 N HCl. The 1 N HCl extract was made to alkaline with conc. NaOH and basic products were taken up in ether. Evaporation of the solvents yielded the acid-neutral fraction and the basic fraction, respectively.

**Isolation of Trp-P-1 and Trp-P-2 (Chart 2)**——Pyrolysis of Tryptophan: D,L-Tryptophan (250 g) was placed in a one-liter Pyrex flask with a side arm and the flask was heated on a direct flame. Volatile products were collected in two traps cooled in ice. The black tar (162 g), whose mutagenic activity to TA98 in the presence of S-9 mix was 2242 revertants per 100 μg per plate, was obtained.

Fractionation of Pyrolysate: The tar (650 g) obtained from a total 1 kg of tryptophan (4 butches) was dissolved in 5 liters of ether and extracted two times first with one liter of 6 N HCl and then one liter of 1 N HCl, successively. The remaining ether solution contained acid and neutral products (331 g) (54 revertants/100 μg/plate). After the aqueous solution was neutralized by $Na_2CO_3$ and adjusted to alkaline by 6 N NaOH, the alkaline aqueous solution saturated with NaCl was extracted with ether, to give basic products (158 g) (3520 revertants/100 μg/plate).

Silica Gel Chromatography (Fig. 1): A portion (50 g) of the basic fraction was dissolved in AcOEt and was chromatographed over a silica gel column (4.5 × 96 cm) using AcOEt to get fractions No. [I]—1—30 of each 200 ml, and AcOEt–MeOH (7: 3) to fractions No. [I]—31—40 of each 200 ml as solvents. A large quantity of a mixture of harman and norharman was obtained from the fractions No. [I]—16—20 as crystal after evaporation of the solvent. From the fract. No. [I]—34—40, tryptamine was obtained. Mutagenic substance was eluted in fractions No. [I]—32—33 (1194 mg) (6000 revertants/100 μg/plate).

11) *a*) K. Takeda, T. Ohta, K. Shudo, T. Okamoto, K. Tsuji, and T. Kosuge, *Chem. Pharm. Bull.* (Tokyo), **25**, 2145 (1977); *b*) H. Akimoto, A. Kawai, H. Nomura, M. Nagao, T. Kawachi, and T. Sugimura, *Chem. Letters.*, **1977**, 1061.

Alumina Chromatography (Fig. 2): The active fractions No. [I]—32—33 (969 mg) was fractionated into 37 fractions by chromatography on an alumina column (2 × 42 cm) using AcOEt to fractions No. [II]—1—20 of each 50 ml, AcOEt–MeOH (7: 3) to fractions No. [II]—31—36 of each 50 ml and MeOH (300 ml/fractions No. [II]—37) as solvents successively, to give active fractions No. [III]—32—36 (160 mg) (2100 revertants/μg/plate).

CM-Sephadex C-25 Chromatography (Fig. 3): The fractions No. [II]—32—36 (160 mg) was purified by chromatography on a CM-Sephadex C-25 column (1.5 × 20 cm). After thoroughly washing with MeOH–$H_2O$ (3: 1, 600 ml), the active fractions No. [III]—11—17 (60 mg) (5100 revertants/μg/plate) was eluted with MeOH–2 N AcOH (3: 1).

Gel Filtration on Sephadex LH-20 (Fig. 4): Fractions No. [III]—11—17 (60 mg) was further purified by gel filtration on Sephadex LH-20 (1.5 × 65 cm) using MeOH as solvent and fraction of each 2.2 ml was fractionally collected. From the eluate of the first peak fractions No. [IV]—10—11, 5 mg of Trp-P-1 (6300 revertants/μg/plate) was isolated after recrystallization from acetone–MeOH.

CM-Sephadex C-25 Chromatography: The eluate of the second peak fractions No. [IV]—31—40 was rechromatographed on CM-Sephadex C-25 (1.5 × 10 cm) using MeOH–0.8 N AcOH (3: 1) as solvent to give Trp-P-2 (10 mg) (14000 revertants/μg/plate).

**Physical Properties of Trp-P-1 and Trp-P-2**——Trp-P-1 Acetate: Pale brown colored needles or small prisms. mp 252—262° (from AcOEt). The crystal form changed at about 200° Dragendorff reagent: positive. NMR ($CD_3OD$) $\delta$: 1.18 (3H), 2.11 (3H), 2.68 (3H), 6.95—7.28 (3H, multiplet), 7.75 (1H, doublet). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3360, 3100, 1655, 1405, 1340, 1265, 1238, 925, 825, 775, 754, 743. MS *m/e*: 211 ($M^+$), 193, 183, 169, 168, 167, 166, 142, 140, 115, 60. UV $\lambda_{max}^{MeOH}$ nm: 245 (sh), 265, 267 (sh), 292, 305, 317. *Anal.* Calcd. for $C_{13}H_{13}N_3 \cdot CH_3COOH$: C, 66.40; H, 6.32; N, 15.49. Found: C, 66.47; H, 6.40; N, 15.04.

Trp-P-2 Acetate: Brown colored needles. mp 248—250° (from AcOEt–MeOH). Dragendorff reagent: positive. NMR ($CD_3OD$) $\delta$: 1.96 (3H), 2.76 (3H), 6.35 (1H, singlet), 7.00—7.40 (3H, multiplet), 7.80 (1H, doublet). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3250, 3020, 2800—2400, 1670, 1655, 1615, 1540, 1462, 1403, 1340, 1268, 1205, 842, 734. MS *m/e*: 197 ($M^+$), 180, 179, 170, 169, 157, 155, 127. UV $\lambda_{max}^{MeOH}$ nm: 244 (sh), 265, 268 (sh), 290—330 (broad). UV $\lambda_{max}^{MeOH+1N\ NaOH}$ nm: 241, 262, 290—330 (broad). *Anal.* Calcd. for $C_{12}H_{11}N_3 \cdot CH_3COOH \cdot 2H_2O$: C, 57.32; H, 6.53; N, 14.33. Found: C, 57.71; H, 6.52; N, 14.13.