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Induction of DNA Recombination by Activated 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

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To investigate the genotoxic properties of a food-derived carcinogen, 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole (Trp-P-2), we have tested whether Trp-P-2 and its metabolically transformed products can induce DNA recombinations. Trp-P-2 is a strong mutagen and its activated form, the *N*-hydroxylated derivative, Trp-P-2(NHOH), is known to form DNA adducts and cause DNA chain cleavage. Using a system in which phage λ undergoes recombination inside host *Escherichia coli*, we have found that Trp-P-2(NHOH), but not Trp-P-2 itself, can induce recombination. A nitroso derivative of Trp-P-2, Trp-P-2(NO), which can be reduced intracellularly to form Trp-P-2(NHOH), also induced recombination. Active oxygens are implicated in this recombinogenic action, since Trp-P-2(NHOH) is known to undergo spontaneous oxidative degradation, generating active oxygen radicals which can cause DNA chain cleavages. 4-Hydroxyaminoquinoline *N*-oxide and phenylhydroxylamine also showed recombinogenic actions in this assay system; hence, it is suspected that aromatic amine-type carcinogens have this property in common.

Key words: Trp-P-2 — DNA strand break — Recombination — Bacteriophage λ

Trp-P-2[7] is one of the carcinogenic heterocyclic amines present in cooked foods.[1)] Trp-P-2 is metabolically converted first into its *N*-hydroxylated form, Trp-P-2-(NHOH), which is then converted into its *O*-esterified derivatives. These activated forms of Trp-P-2 can react with DNA and form base adducts.[2-4)] In recent studies, we found interesting properties of the activated Trp-P-2. Trp-P-2(NHOH) is unstable in neutral aqueous solutions, undergoing oxidative degradation to form superoxide anion and other active oxygen species.[5)] Furthermore, Trp-P-2(NHOH) generates active oxygens in cells, when given externally.[6)] The nitroso derivative of Trp-P-2, Trp-P-2(NO), is formed during oxidative degradation *in vitro*,[5)] and Trp-P-2(NO) can be reduced by the action of biological reductants or superoxide anion to regenerate Trp-P-2(NHOH).[5,7)] The Trp-P-2(NHOH)-induced active oxygen species can cause DNA single-strand breaks in both naked and intracellular DNA.[8)]

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[7] The abbreviations used are: Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; 2-AF(NHOH), 2-hydroxyaminofluorene; 4HAQO, 4-hydroxyaminoquinoline *N*-oxide; Trp-P-2-(NHOH), 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2(NO), 3-nitroso-1-methyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2(NO_2), 3-nitro-1-methyl-5*H*-pyrido[4,3-*b*]indole; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 4NQO, 4-nitroquinoline *N*-oxide; PAMBC, phthalic acid mono-*n*-butyl ester cupric salt; SCE, sister chromatid exchange; SOD, superoxide dismutase; UV, ultraviolet.

Here we have investigated possible biological consequences of the DNA strand breaks inducible by Trp-P-2-(NHOH). Meselson and Radding proposed a model for the initiation of DNA recombination by single-strand breaks.[9)] Furthermore, any other possible recombination model should require DNA strand cleavage in the initiation step forming the Holliday structure, or in the completion step resolving it. Therefore, it is of interest to know whether DNA-cleaving agents can induce recombination. In this study, we investigated the ability of Trp-P-2(NHOH) and other Trp-P-2 derivatives to induce phage λ DNA recombination. We also investigated the activity of several other DNA-damaging compounds. The relationship between the activities inducing DNA recombination and damage is discussed.

MATERIALS AND METHODS

Bacterial strains and phages Bacterial strains used as hosts for phage λ were *E. coli* Ymel *supF58*, 594 sup^0, and BIK1206 *lexA3* (ind^-). Phage λ strains used were λDF; λ *imm434cI Dam15 FIam96B b538 red3*, and λSR; λ *imm434cI Sam7 Ram5 int6 red3*.

The bacterial strains used as the hosts for phage cross experiments are all recombination-proficient, and the phages, λDF and λSR, are deficient in the viral recombination system due to the genotypes $red3^-$ and int^-. Therefore, the recombination observed in this study is mediated solely by the bacterial recombination machinery.

Chemicals Trp-P-2 acetate, MNNG, bleomycin and hydrogen peroxide were obtained from Wako Pure Chemical (Osaka), 4HAQO and PAMBC from Tokyo Kasei (Tokyo), SOD [EC 1.15.1.1] of bovine erythrocytes from Sigma (St. Louis, MO, USA), and chlorophyllin from Nacalai Tesque (Kyoto). 2-AF(NHOH) was a generous gift from Dr. Ryuichi Kato of Keio University, Medical School. Trp-P-2(NHOH), Trp-P-2-(NO), Trp-P-2(NO_2) and phenylhydroxylamine were prepared as described previously.[5]

Measurement of recombination frequency Recombination frequency was measured by means of a cross experiment as follows. *E. coli* Ymel or BIK1206 cells were grown at 37°C in λ broth, which contained 10 g of polypepton and 2.5 g of NaCl in 1 liter. The cells were harvested at the early log phase ($1-3 \times 10^8$ cells/ml) and suspended in 10 m*M* $MgSO_4$ at a density of 10^9 cells/ml. The bacteria were mixed with λDF and λSR simultaneously at a multiplicity of infection of 5, for individual strains of phages, and the adsorption was done at 37°C for 10 min. After the adsorption, the bacterial cells were washed twice with 10 m*M* Tris-HCl (pH 7.5)-10 m*M* $MgSO_4$ and diluted into λ broth pre-warmed at 37°C at a density of 10^4 cells/ml. Immediately, a test compound was added to the broth and the mixture was shaken at 37°C. After 1 or 2 h of shaking, a few drops of chloroform were added to the culture for the complete lysis of infected cells. Total progeny phages were counted by plaque formation on a lawn of Ymel, and the number of wild-type phages resulting from recombination was determined by plaque formation on a lawn of *E. coli* 594. The culture was appropriately diluted to produce more than 100 plaques on each plate and averages of duplicate plates were recorded. The recombination frequency was expressed as the fraction of wild-type phages in total phages.

UV irradiation A single 15-W UV lamp (Hitachi, Tokyo), which emits predominantly 254-nm light, was used for UV irradiation of infected *E. coli*. The *E. coli* infected with λDF and λSR was suspended in M9 buffer and irradiated. The bacteria were then diluted into λ broth at 37°C and after incubation the daughter phages were titrated as described above. The dose of UV light was measured with a Topcon UV radiometer (Tokyo Kogaku Kikai, Tokyo).

RESULTS

Induction of recombination between λ DNAs Ymel, a suppressor-positive strain of *E. coli*, was infected with a mixture of two λ phage mutant strains, λDF and λSR. If their DNAs replicating in a cell recombine in the manner shown in Fig. 1, the recombination should generate a wild-type progeny phage, λwild, that can form plaques on *E. coli* 594, a strain deficient in the suppressor gene. A control cross experiment showed the background level: about 1% of total progeny phages had wild-type genes of *D*, F_I, *S* and *R*. The fraction of recombinant phage population among total phages increased up to 5 times the background level on addition of Trp-P-2(NHOH), and the increase was dependent on the dose of Trp-P-2-(NHOH) (Fig. 2).

Trp-P-2(NO), which is incapable of cleaving naked DNA but can induce DNA strand breaks in intracellular DNA (Y. Wataya *et al.*, unpublished results), increased the recombination frequency, and the recombination-inducing ability was half as potent as that of Trp-P-2-(NHOH) (Fig. 2). Trp-P-2 and Trp-P-2(NO_2), which cannot cause any DNA strand breaks either *in vitro* or *in vivo*, showed no recombination-inducing ability (Fig. 2). These results suggest that the DNA-cleaving activities of these agents are associated with the induction of DNA recombination.

Activated Trp-P-2 has been reported to induce SOS function in *E. coli*.[10] The induction of recombination observed here might be caused as a result of an overproduction of RecA protein, which is an initial step of DNA lesion-mediated bacterial SOS function. To explore this possibility, we used an SOS-deficient strain BIK 1206; *lexA* (*ind*$^-$) as the host bacteria. Here also the induction of recombination was observed: without Trp-P-2(NHOH), 5.5%; with 3 μ*M* Trp-P-2(NHOH),

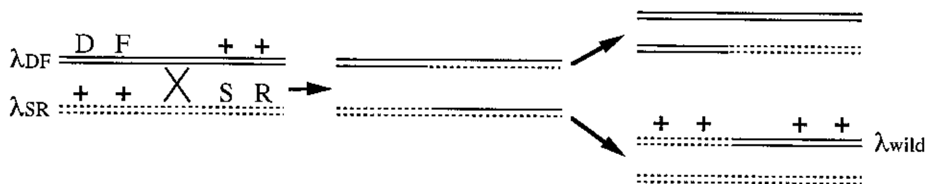

Fig. 1. Recombination between λDF DNA and λSR DNA resulting in λwild DNA. λDF carries amber mutations in genes *D* and F_I. λSR carries amber mutations in genes *S* and *R*. These mutant genes are indicated as *D*, *F*, *S* and *R*. Genes *S* and *R* of λDF and genes *D* and F_I of λSR are wild type, which are marked with +. Recombination between λDF and λSR generates λwild, which has wild-type genes of *D*, F_I, *S* and *R*.

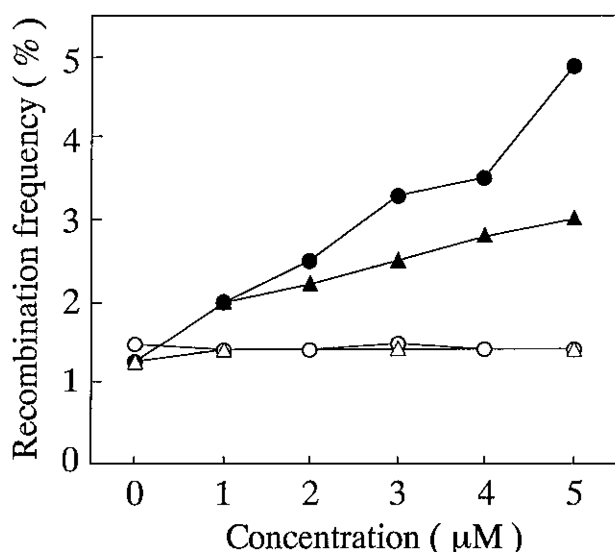

Fig. 2. Effects of Trp-P-2 derivatives on the recombination frequency between λDF and λSR in *E. coli* Ymel. *E. coli* Ymel propagating λDF and λSR simultaneously was incubated in λ broth containing Trp-P-2(NHOH) (●), Trp-P-2-(NO) (▲), Trp-P-2(NO_2) (△) or Trp-P-2 (○) at the concentrations indicated. After lysis, daughter phages were titrated on *E. coli* Ymel for total phages and on *E. coli* 594 for wild-type phages. Recombination frequencies were calculated from the number of wild-type phages divided by the number of total phages.

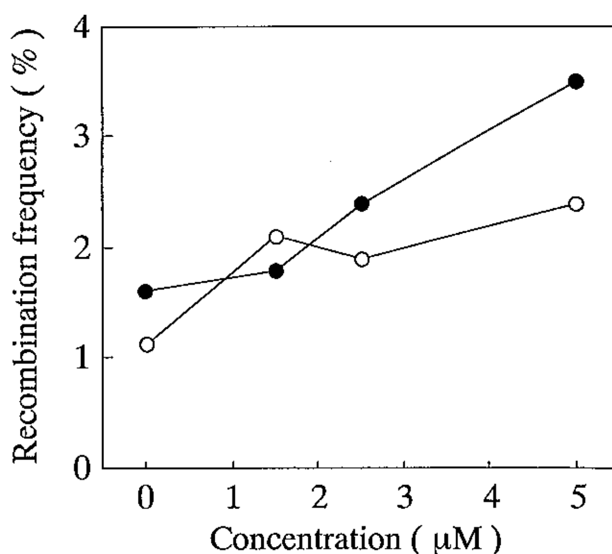

Fig. 3. Effects of phenylhydroxylamine and 4HAQO on the recombination frequency in *E. coli* Ymel. *E. coli* Ymel propagating λDF and λSR simultaneously was incubated in λ broth containing phenylhydroxylamine (●) or 4HAQO (○) at the concentrations indicated. Recombination frequency was measured as described in the legend to Fig. 2.

12.5%; and with 5 μM Trp-P-2(NHOH), 22.5%. Thus, the recombination frequency reached a level about 5-fold greater than the control, the same level as that observed with Ymel. This result indicates the independence of the induction of the recombination from SOS function. In other words, there seems to be no need of SOS signals from damaged DNA for the substrate DNAs to be recombined.

Two other mutagenic aromatic hydroxylamines, phenylhydroxylamine and 4HAQO, were examined for effect on the recombination frequency in *E. coli* Ymel. They also have the abiliy to cleave purified DNA (data not shown). Both of the compounds elevated the frequency to a value twice as high as that of the control (Fig. 3).

UV light caused induction of DNA recombination in Ymel to 8.4% at a dose of 4.2 mJ/cm^2. UV is known to produce pyrimidine dimers and other types of damage in DNA bases. The damaged bases may be removed by cellular excision repair machinery. The nicks introduced by the excision nuclease or by UV-induced base damage itself may participate in the induction of the recombination. MNNG, a mutagenic methylating agent, also stimulated the recombination to the extent of 3.6% at 50 μM.

Table I. Modulation of Trp-P-2(NHOH)-mediated Recombination by Reagents

Reagents (concentration)[a]	Relative recombination frequency (%)
Trp-P-2(NHOH) (5 μM)	100
+ Chlorophyllin (1 mM)	7
+ L-Ascorbic acid (1 mM)	50
+ Cysteamine (1 mM)	85
+ PAMBC (0.1 mM)	91
+ SOD (20 μg/ml)	108

a) Each modulator and Trp-P-2(NHOH) were added at the same time. The recombination frequencies were measured as described in "Materials and Methods."

The bases methylated by MNNG may be excised and nicks introduced may play a role here, too. MNNG was reported to induce DNA strand breaks in intracellular DNA.[11]

2-AF(NHOH), another mutagenic aromatic hydroxylamine, did not induce recombination (data not shown). 2-AF(NHOH) is extremely unstable in neutral aqueous solutions. Therefore, we suspect that the agent did not reach the DNA beyond the bacterial membrane. Hydrogen peroxide (5 mM) and bleomycin (1 μg/ml), which are known to cleave DNA,[12,13] had no effect (data not shown).

Inhibition of the recombination induction by Trp-P-2-(NHOH) The effect of several agents, which might influence the mutagenicity or the DNA-breaking activity of Trp-P-2(NHOH), on the induction of the recombination was examined. The results are shown in Table I. Chlorophyllin can form complexes with Trp-P-2 derivatives and has antimutagenic activities.[14, 15] Chlorophyllin inhibited the induction of recombination almost completely. It may prevent permeation of Trp-P-2(NHOH) into *E. coli* cells by forming a complex. Addition of L-ascorbic acid, an antioxidant and free radical scavenger, suppressed 50% of the induced recombination. This result suggests that free radicals or oxidants generated during the degradation of Trp-P-2(NHOH) participate in the recombination. However, another antioxidant, cysteamine, had no significant effect. SOD, a scavenger of superoxide anion, or a membrane-permeable SOD mimic, PAMBC, was not effective. It should be noted that cysteamine and PAMBC did not inhibit intracellular DNA breaks inducible by Trp-P-2(NHOH) (Y. Wataya *et al.*, unpublished results). SOD may be unable to enter bacterial cells.

DISCUSSION

We have demonstrated here that DNA recombination in *E. coli* cells was induced by direct mutagens that have DNA-cleaving activity. Single-strand breaks in phage DNA, which possibly took place during these treatments, would have stimulated the recombination, as the Meselson-Radding model predicts[9]; in this model, DNA single-strand breaks initiate the recombination. The breaks might be induced by active oxygens derived from the auto-degradation of Trp-P-2(NHOH). However, two other DNA-cleaving agents, bleomycin and hydrogen peroxide, did not induce DNA recombination. A DNA strand break alone might not be sufficient for stimulating recombination.

In our system, UV irradiation also induced recombination. It has been reported that recombination of viral DNA in mammalian cells is stimulated by UV damage.[16–18] Bhattacharyya *et al.* pointed out that in the case of UV, the activity of excision repair of the damage can prevent the induction of recombination, and they proposed that unexcised DNA lesions lead to recombination.[16] This suggests that the recombination is not induced by strand scissions *via* the action of repair endonucleases, but by base modification. In our case, single-strand breaks as well as Trp-P-2 adducts in DNA could have affected the recombination. Further work is necessary to evaluate which of these two different lesions is relevant to the induction.

The recombination observed here was not dependent on SOS response. More precisely, it did not require the presence of SOS-inducible proteins that are produced *via* inactivation of the LexA protein. The background level of recombination in BIK1206 was somewhat higher than that in Ymel. Wild-type LexA protein might also be inhibitory to the recombination. Further experiments are needed to confirm this, because the two strains used here do not have the same genetic background.

In mammalian cells, DNA recombination may sometimes be the cause of chromosome aberrations such as SCE (sister chromatid exchange), chromosome translocation and chromosome deletion. Chromosomal translocations[19–23] and deletions[24] have been frequently identified in tumor tissues. Tohda *et al.*[25] reported that SCE is inducible by activated Trp-P-2 and 4NQO; 4NQO is known to be reduced to 4HAQO in the cells. These facts suggest that DNA recombination could play some role in the carcinogenesis by Trp-P-2 and 4NQO. It is unclear whether mammalian cells are equipped with a response system similar to the bacterial SOS function. The SOS function might be a process specific to bacteria. The induction of recombination by Trp-P-2(NHOH) observed here did not require SOS response. Thus, the mechanism of recombination reported here could be relevant to the recombination in mammalian cells.

The assay employed in this study is simple and the results can be obtained within 24 h. It might be useful for screening chemicals for their DNA recombination-inducing activity, a factor important in evaluating the genotoxicity of an agent.

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REFERENCES

1) Sugimura, T. Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat. Res.*, **150**, 33–41 (1985).
2) Hashimoto, Y., Shudo, K. and Okamoto, T. Activation of a mutagen, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole. Identification of 3-hydroxyamino-1-methyl-5*H*-pyrido-[4,3-*b*]indole and its reaction with DNA. *Biochem. Biophys. Res. Commun.*, **96**, 355–362 (1980).

3) Mita, S., Ishii, K., Yamazoe, Y., Kamataki, T., Kato, R. and Sugimura, T. Evidence for the involvement of *N*-hydroxylation of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole by cytochrome P-450 in the covalent binding to DNA. *Cancer Res.,* **41,** 3610–3514 (1981).
4) Kato, R. and Yamazoe, Y. Metabolic activation and covalent binding to nucleic acids of carcinogenic heterocyclic amines from cooked foods and amino acid pyrolysates. *Jpn. J. Cancer Res.,* **78,** 297–311 (1987).
5) Hiramoto, K., Negishi, K., Namba, T., Katsu, T. and Hayatsu, H. Superoxide dismutase-mediated reversible conversion of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]-indole, the *N*-hydroxy derivative of Trp-P-2, into its nitroso derivative. *Carcinogenesis,* **9,** 2003–2008 (1988).
6) Wataya, Y., Yamane, K., Hiramoto, K., Ohtsuka, Y., Okubata, Y., Negishi, K. and Hayatsu, H. Generation of intracellular active oxygens in mouse FM3A cells by 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole, the activated Trp-P-2. *Jpn. J. Cancer Res.,* **79,** 576–579 (1988).
7) Saito, K., Yamazoe, Y., Kamataki, T. and Kato, R. Activation and detoxication of *N*-hydroxy-Trp-P-2 by glutathione and glutathione transferases. *Carcinogenesis,* **4,** 1551–1557 (1983).
8) Wakata, A., Oka, N., Hiramoto, K., Yoshioka, A., Negishi, K., Wataya, Y. and Hayatsu, H. DNA strand cleavage *in vitro* by 3-hydroxyamino-1-methyl-5*H*-pyrido-[4,3-*b*]indole, a direct-acting mutagen formed in the metabolism of carcinogenic 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole. *Cancer Res.,* **45,** 5867–5871 (1985).
9) Meselson, M. S. and Radding, C. M. A general model for genetic recombination. *Proc. Natl. Acad. Sci. USA,* **72,** 358–361 (1975).
10) Ohta, T., Nakamura, N., Morita, M., Shirasu, Y. and Kada, T. The SOS-function-inducing activity of chemical mutagens in *Escherichia coli. Mutat. Res.,* **131,** 101–109 (1984).
11) Haggerty, H. G., Kim, B.-S. and Holsapple, M. P. Characterization of the effects of direct alkylators on *in vitro* immune responses. *Mutat. Res.,* **242,** 67–78 (1990).
12) Schraufstätter, I., Hyslop, P. A., Jackson, J. H. and Cochrance, C. G. Oxidant-induced DNA damage of target cells. *J. Clin. Invest.,* **82,** 1040–1050 (1988).
13) Stubbe, J. and Kozarich, J. M. Mechanisms of bleomycin-induced DNA degradation. *Chem. Rev.,* **87,** 1107–1136 (1987).
14) Negishi, T., Arimoto, S., Nishizaki, C. and Hayatsu, H. Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2). *Carcinogenesis,* **10,** 145–149 (1989).
15) Negishi, T., Nakano, H., Kitamura, A., Itome, C., Shiotani, T. and Hayatsu, H. Inhibitory activity of chlorophyllin on the genotoxicity of carcinogens in *Drosophila. Cancer Lett.,* **83,** 157–164 (1994).
16) Bhattacharyya, N. P., Maher, V. M. and McCormick, J. J. Effect of nucleotide excision repair in human cells on intrachromosomal homologous recombination induced by UV and 1-nitrosopyrene. *Mol. Cell. Biol.,* **10,** 3945–3951 (1990).
17) Narin, R. S., Humphrey, R. M. and Adair, G. M. Transformation depending on intermolecular homologous recombination is stimulated by UV damage in transfected DNA. *Mutat. Res.,* **208,** 137–141 (1988).
18) Rainbow, A. J. and Castillo, J. E. Homologous recombination of adenovirus DNA in mammalian cells: enhanced recombination following UV-irradiation of the virus. *Mutat. Res.,* **274,** 201–230 (1992).
19) Boehm, T. and Rabbits, T. H. The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors. *FASEB J.,* **3,** 2344–2359 (1989).
20) Croce, C. M. and Nowell, P. C. Molecular genetics of human B cell neoplasia. *Adv. Immunol.,* **38,** 245–274 (1986).
21) Rowley, J. D. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. *Nature,* **243,** 290–293 (1973).
22) Tsujimoto, Y. and Croce, C. M. Analysis of the structure, transcripts, and protein products of *bcl-2,* the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci. USA,* **83,** 5234–5238 (1986).
23) Weitberg, A. and Corvese, D. Translocation of chromosome 16 and 18 in oxygen radical-transformed human lung fibroblasts. *Biochem. Biophys. Res. Commun.,* **169,** 70–74 (1990).
24) Ponder, B. Gene losses in human tumors. *Nature,* **335,** 400–402 (1988).
25) Tohda, H., Oikawa, A., Kawachi, T. and Sugimura, T. Induction of sister-chromatid exchanges by mutagens from amino acid and protein pyrolysates. *Mutat. Res.,* **77,** 65–69 (1980).