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Chemistry of Mutagens and Carcinogens in Broiled Food

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From a chemical point of view, the following subjects are important areas in studies on mutagens and carcinogens in broiled foods. In addition to heterocyclic amines which need microsomal activation, the structural elucidation of more labile direct-acting mutagens is necessary. It is known that there are still various unknown minor mutagens in broiled foods. Although the structural characterization of such compounds is more difficult, it is important since they might be hazardous in spite of their low mutagenicity. A more feasible and easier method for quantitative analysis of mutagens, in addition to HPLC and GC/MS methods presently employed, must be developed. The mechanism of formation of mutagens by broiling of food should be studied. An effective chemical method to prevent formation of mutagens or to destroy them, once formed, should be developed.

Introduction

In 1978, when we started to isolate and characterize structures of mutagens present in real broiled foods rather than pure amino acid pyrolyzates, we thought that the goals would be difficult to attain. It was expected that many different types of mutagens would be produced during broiling of foods, because components present in foods are quite complex. Contrary to such a pessimistic assumption, we were able to identity three potent mutagens, IQ, MeIQ, and MeIQx, from broiled fish and/or broiled beef (*1–3*). They seemed to be three major mutagens present in broiled foods; however, it is also true that there must be many uncharacterized mutagens in broiled foods in addition to IQ, MeIQ, and MeIQx. An example in support of the above conclusion is shown in Figure 1, which illustrates the chromatographic profile of mutagens in a crude extract of broiled sardines fractionated by high-performance liquid chromatography (HPLC) (*4*). There are many peaks with mutagenic activity in the chromatogram, indicating that there are at least 10 or more mutagenic components in the broiled sardines.

Important Areas in Studies Related to the Chemistry of Mutagens

As far as chemistry is concerned, there are the following subjects concerning mutagens in cooked food: development of a more feasible and easier method for quantitative analysis of mutagens; structure elucidation of new mutagens in broiled food; search for direct mutagens that do not need microsomal activation; mechanism of formation of mutagens by broiling of food; and development of an effective chemical method to prevent formation of mutagens or to destroy them.

Development of a More Feasible and Easier Method for Quantitative Analysis of Mutagens

It is important to quantify amounts of mutagens whose structures are already known in a variety of cooked foods. For studies on mechanism of formation of mutagens and estimation of human health risk for each food, etc., quantitative analysis of actual amounts of each mutagen rather than analyzing total mutagenicity in broiled foods is necessary. There have been several methods previously reported for analysis of mutagenic or carcinogenic heterocyclic amines, such as IQ, MeIQ, and MeIQx isolated from broiled foods, and Trp-P-1, Trp-P-2, Glu-P-1, and α-carbolines isolated from amino acid or protein pyrolyzates (*5–7*). The methods used at present have several drawbacks as follows: they are generally time-consuming or require large amounts of the compounds to be analyzed.

A more serious problem with these methods, in most cases, is the difficulty of obtaining accurate quantitative values, because quite a large portion of mutagens is lost. Also, no exact estimation of yield in purification steps is possible during successive purification procedures re-

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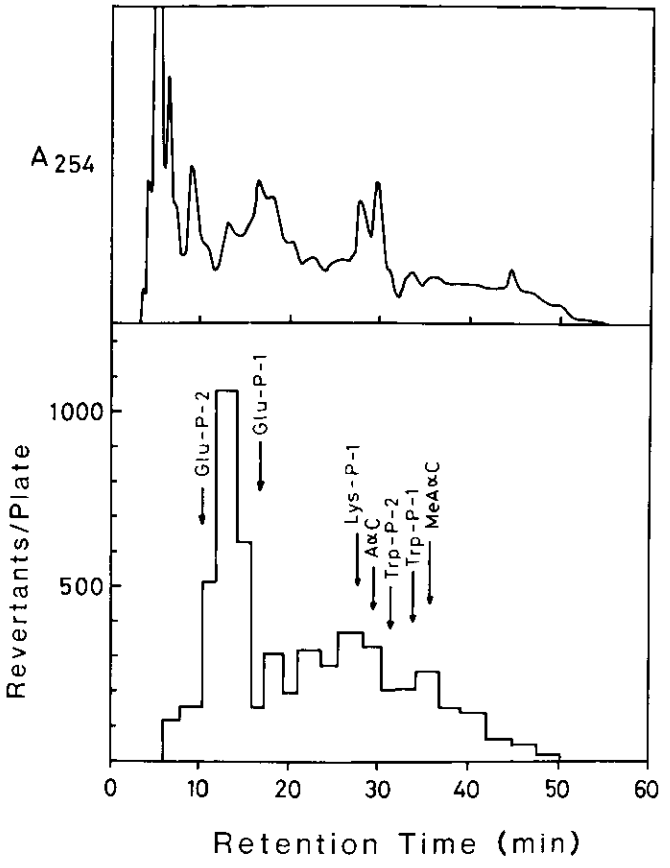

FIGURE 1. Fractionation of mutagens from broiled sardines (silica gel eluate) by HPLC. A sample (1.5 mg) of the silica gel eluate was suspended in 0.1 mL of a solution of 10% methanol–0.04% formic acid. The insoluble residue was removed by centrifugation, and the supernatant was injected into a column of μBondapak/C-18 (0.4 × 30 cm); temperature, ambient; speed 1 mL/min; eluent, (low) 10% methanol–0.04% formic acid, (high) 80% methanol–0.04% formic acid, linear gradient. The elution positions of authentic samples are indicated by arrows. *Salmonella typhimurium* TA 98, a frame shift-sensitive mutant was used for mutation assay.

quired prior to final analysis. A typical scheme for the purification of IQ and MeIQ from broiled sardines from earlier studies is shown in Table 1. Although IQ and MeIQ are thought to be major components in broiled sardines, their final yield in terms of mutagenicity is quite low. Extraction and purification procedures for mutagenic heterocyclic amines generally used at present consist of extraction of cooked food with acidic aqueous solution, organic solvent extraction, acid-base partition, blue-cotton adsorption, column chromatography (Sephadex LH-20, XAD-2, silica gel, etc.), and HPLC. Among those procedures, the blue cotton adsorption technique developed by Hayatsu, which is described separately in these proceedings (*8*), seems to be very powerful for specific isolation of mutagens having planar heterocyclic structures. It should be mentioned, however, that even this procedure is not infallible, because many nonmutagenic compounds present in crude extracts of cooked foods are also absorbed (*9*).

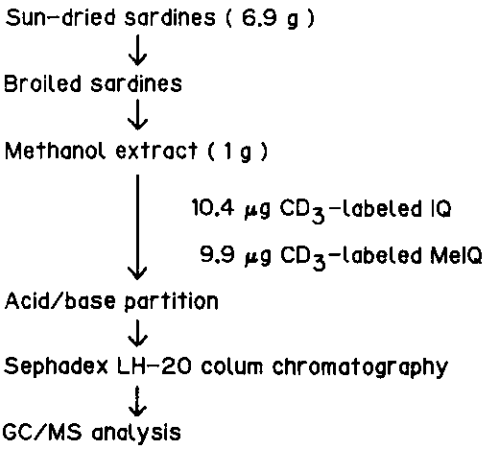

FIGURE 2. Scheme for analysis of IQ and MeIQ in broiled sardines by isotope dilution GC/MS.

Several advanced methods have been introduced recently for quantitative analysis of mutagens in broiled foods. For example, identification of IQ in beef extract was successfully carried out by HPLC coupled with scanning of UV spectra using a photodiode array detector, by Tannenbaum and his co-workers (*10*). Takahashi et al. (*11*) analyzed amounts of IQ, MeIQ, and MeIQx in beef extract and cooked beef by adopting a sensitive electrochemical detection method. Other types of fluorescent carcinogenic heterocyclic amines were analyzed by fluorometry (*11*). Identities of the mutagens were confirmed by their retention time in at least two different HPLC systems. In order to estimate recovery of mutagens during fractionation, ^{14}C-labeled IQ was added prior to fractionation, and the radioactivity at the final stage of purification was counted. With this

Table 1. Purification of mutagens from broiled sardines.

	Weight, mg	Specific activity, revertant/μg[a]	Recovery, %
Methanol extract	817,000	0.3	100
Ether-water partition	537,000	0.2	40.5
HP-20 column chromatography	54,000	1.0	22.3
Chloroform-methanol-water partition	16,600	3.5	24.7
Sephadex LH-20 column chromatography	89.4	243	9.2
Silica gel column chromatography	4.6	598	1.2
HPLC			
IQ	0.06	8,000	0.2
MeIQ	0.04	18,500	0.4

[a] Mutagenic assay with TA98 using S9 mix.

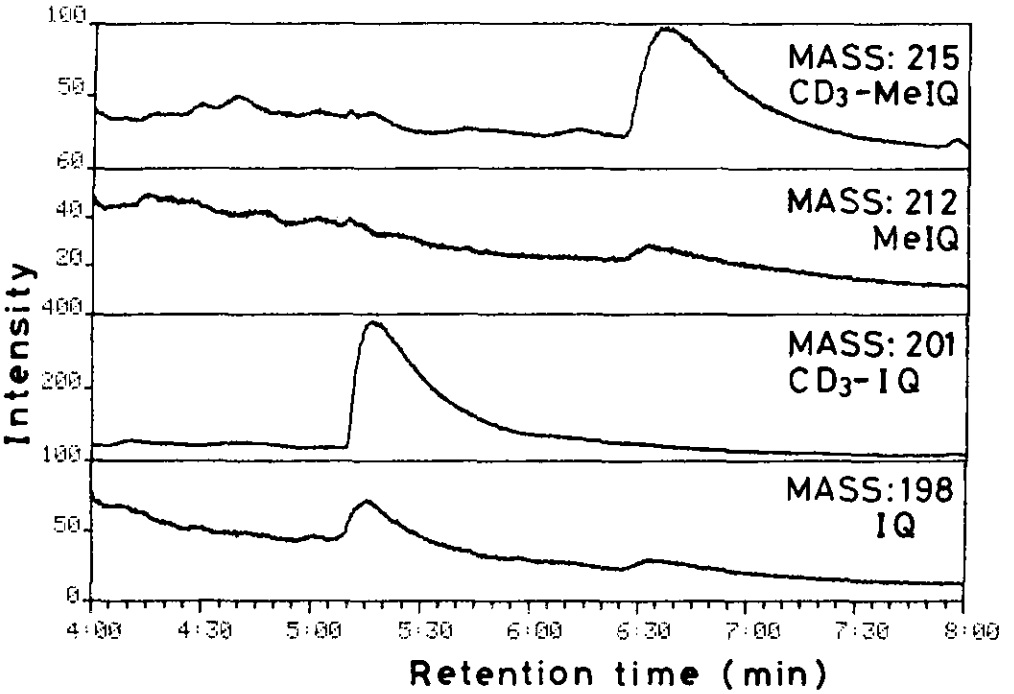

FIGURE 3. Quantitative analysis of IQ and MeIQ in broiled sardines by GC/MS using isotope-labeled internal standard with selected ion monitoring. Instrument: Varian MAT 44S GC/MS system. A 6-m capillary column coated with SP-2100 was used under the following conditions: initial temperature; 180°C, final temperature; 250°C, increasing temperature rate; 20°C/min.

method, however, it is not certain whether or not the same extent of recovery is obtained for other mutagens present at concentrations different from that of ^{14}C-labeled IQ. The procedures described above could be used for samples in which concentrations of the mutagens are relatively high, such as beef extract or cooked beef. As shown in Table 1, the content of IQ and MeIQ in broiled sardines is extremely small ($\sim 10^{-6}\%$). Therefore, in the case of crude extracts of cooked foods such as broiled sardines, it is still extremely difficult to observe well-separated peaks for the mutagenic heterocyclic amines in the chromatogram during the final HPLC step.

Another method, which should be superior to HPLC in terms of sensitivity and selectivity is GC/MS using isotope-labeled internal standards. For this purpose, we chemically synthesized CD_3-labeled IQ, MeIQ, and MeIQx. In the case of quantitative analysis of IQ and MeIQ in broiled sardines, appropriate amounts of CD_3-labeled IQ and MeIQ were added to the initial methanol extract, prior to fractionation (see Fig. 2). The final preparation was analyzed by capillary GC/MS, monitoring molecular ions of both natural and CD_3-labeled compounds. Amount of the natural compound can be calculated from the ratio of each peak area (Fig. 3). With this technique, yield of the mutagens during the course of fractionations does not influence the result (*12*). The method, however, also has the following drawback: even after Sephadex LH-20 column chromatography, contents of IQ and MeIQ are 0.1 to 0.01% of the total material. Injection of such a crude sample into a capillary GC column leads to deterioration of the column, thus making it difficult to obtain reproducible results. Perhaps the most sophisticated advanced method for the analysis of mutagens in broiled foods is directly coupled liquid chromatography/mass spectrometry using isotope-labeled internal standards. Details of this method are described by McCloskey (*13*). With this method, a larger amount of sample can be loaded onto a reversed phase column without causing its deterioration. Identification of the compounds is made by simultaneous measurement of retention time and molecular ion.

Problems of Isolation of New Mutagens from Broiled Foods, and a New Approach for their Solution

As shown in Table 1, final yields of IQ and MeIQ from broiled sardines are quite low when compared with total mutagenicity present in the initial crude extract. It is likely that some unknown mutagens are lost during sample work-up due to their instability. Such instability might be a more serious problem in the case of isolation of direct mutagens, which do not require microsomal activation for mutagenicity, because they already possess a reactive group, and therefore are more easily

Compound	Revertants/nmole	Compound	Revertants/nmole
IQ	85,700		55
MeIQ	140,000		79
	30,100		6
	159,000		
	98,100		

FIGURE 4. Mutagenicities of IQ and its derivatives.

inactivated. It is important to characterize structures of direct mutagens in broiled foods, when their possible relationship to stomach cancer is considered.

IQ, MeIQ, and MeIQx isolated so far from broiled food possess quite high specific activities of mutagenicity, when assayed by Ames' bacterial mutation assay. In fact, MeIQ shows the highest mutagenicity (600,000 revertants/μg) among the mutagens so far examined using *Salmonella* TA 98 as a test strain. It does not necessarily mean that a compound having strong mutagenicity in a bacterial mutation assay possesses strong carcinogenicity, because fluctuations on the order of 10^3 to 10^4 in correlation between mutagenicity and carcinogenicity are frequently observed. It is important to look for mutagens whose mutagenicity is not high, because they might be potent carcinogens even though they are not strongly mutagenic. It is very difficult to isolate such weak mutagens from broiled food, if the bacterial mutation assay is used as the screening method, because such weak mutagens might not be seen due to interference overlapped from major mutagenic components which copurify during fractionation procedures. There might be other IQ derivatives in broiled foods, because many chemically synthesized IQ derivatives show much weaker mutagenicity as in Figure 4 (*14*).

Other biological mutation assay methods using eukaryotic cells are generally time-consuming and not practical for a routine assay method. For this reason, we developed a new chemical method to identify mutagens (*15*). The method consists of using a guanosine derivative that contains a fluorescent group, 2′-deoxy-2′-(2″,3″-dihydro-2″,4″-diphenyl-2″-hydroxy-3″-oxo-1″-pyrrolyl) guanosine(abbreviated as FG) to trap the mutagen, because most mutagens and carcinogens are known to react with the guanine residue in DNA and bind covalently. Therefore the fluorecent guanosine derivative can be mixed with a mutagen and incubated, and the reaction mixture fractionated by HPLC with monitoring by a fluorescent detector. When the mutagen reacts with the guanosine derivative, the chromatogram generally shows additional peak(s) as shown in Figure 5. Similarly an isopropylidene derivative of guanosine can be used as a reagent. In that case, the adduct can be easily purified by extraction with organic solvents such as ethyl acetate. Those methods can be applied for identification of both indirect and direct mutagens. In the case of indirect mutagens, the mutagens are converted to active forms by incubation with S9 mix before reacting with the guanine derivative. The sensitivity of those methods is almost comparable to that of the bacterial mutation assay.

As described by Kasai (*16*), we were able to find a new DNA hydroxylation reaction in which 8-hydroxyguanine is formed by oxygen radical-forming reagents which should be present in broiled foods, based on stud-

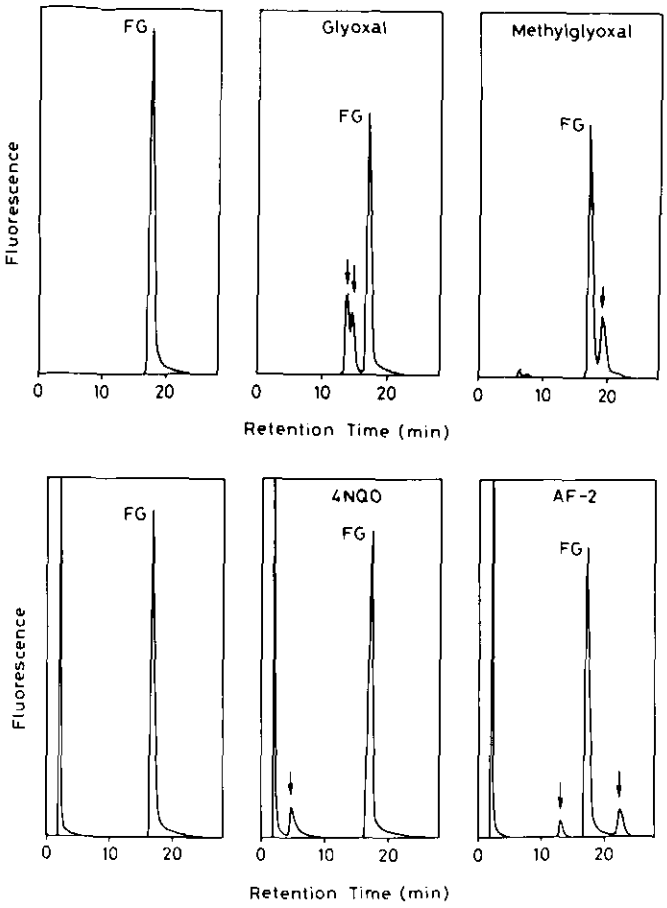

FIGURE 5. Detection of adducts of mutagens with the fluorescent derivative (FG) by HPLC. Mutagens as specified were incubated with FG in the presence or absence of S9 mix and analyzed by HPLC with a fluorescent detector. Column; Merck Hibar column LiChrosorb RP-18, 5μ, 0.4 × 25 cm; solvent, 30% aqueous methanol containing 10 mM ammonium acetate (pH 5.3). Direct-acting mutagens, glyoxal or methylglyoxal (50 μg/100 μL of DMSO), were mixed with 100 μL of FG solution (50 μg/100 μL H_2O) and 500 μL of sodium phosphate buffer (100 mM, pH 6.8). In the experiment with 4NQO and AF-2, 100 μL of solutions of these mutagens (50 μg/100 μL of DMSO) were mixed with 100 μL of solution of FG (50 μg/100 μL H_2O) and 500 μL of S9 mix. (pH 7.4). Peaks of adducts are indicated by arrows.

ies using heated glucose as a model system (*17*). Attempts are now being made to identify new mutagens present in broiled foods by using those techniques.

Conclusion

The present status and problems of isolation, characterization, and quantitative analysis of mutagens in broiled foods have been reviewed. Although several new mutagens, namely IQ, MeIQ, and MeIQx, have been isolated as major components from broiled foods to date, further investigation is necessary in order to determine whether additional mutagens are present in cooked foods in order to obtain a clearer picture regarding the presence of mutagens in food. For this purpose, it is important to develop easier isolation and analytical methodology, and in fact, some new techniques have been developed, as discussed. Isolation and characterization of more new mutagens and their synthesis and risk estimation by using synthetic compounds are the first essential steps for full understanding of the risk to human health of mutagens in cooked foods.

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