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Characterization of the mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline prepared from a 2-methylpyridine/creatinine/acetylformaldehyde model system

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A mixture of 2-methylpyridine, creatinine and aldehydes was heated in diethylene glycol containing 5% water for 1 h at 140°C. The mutagenic compounds were purified by XAD-2 column chromatography, acid/base partition, blue cotton treatment, thin layer chromatography and HPLC. The active substances purified from each step were monitored by their mutagenicity with *Salmonella typhimurium* TA98 in the presence of S9 mix. Among the mutagens collected, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was isolated from HPLC, and was identified by its UV and mass spectrum using a photodiode array detector and mass spectrometry. Our findings appear to be the first experimental evidence to substantiate the hypothetical pathway for the formation of IQ mutagens from a heated model system consisting of a pyridine or pyrazine derivative, an aldehyde and creatinine or creatine.

Introduction

Food is a mixture of large numbers of chemicals such as proteins, amino acids, carbohydrates, lipids, vitamins, creatinine and minerals. These chemicals undergo complex reactions when they are heated. Among the reactions occurring in food during heat treatment, the Maillard reaction plays the most important role in the formation of mutagens (Spingarn and Gravie, 1979; Powrie *et al.*, 1982; Shibamoto *et al.*, 1989). A tentative pathway for the formation of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) compounds via the Maillard reaction was suggested by Jagerstad *et al.* (1983, 1986). It was postulated that the amino-imidazo part of the molecule is formed from creatine by cyclization and water elimination. The remaining parts of the IQ compounds were assumed to arise from Strecker degradation products, e.g. pyridines or pyrazines, formed by the Maillard reaction between hexose and various amino acids. Aldol condensation was thought to link the two parts together via a Strecker aldehyde. This hypothesis was verified using a model system with creatin(in)e, glycine or alanine and glucose, dissolved in diethylene glycol containing 14% water and refluxed at 130°C for 2 h (Jagerstad *et al.*, 1984; Grivas *et al.*, 1986). The mixture showed high mutagenic activity, as compared with the weak mutagenicity, if any, produced by other models of heating two reactants at a time (Grivas, 1986). The addition of synthetic pyridines or pyrazines to the reaction mixture increased the mutagenic activity by ~50% (Jagerstad *et al.*, 1983).

The possible role of the Maillard reaction in the hypothetical formation of IQ compounds has been described in greater detail by Nyhammar (1986). However, although most model systems of thermic mutagen chemistry produce various Ames-positive mutagens, the direct formation and isolation of IQ and/or its derivatives in these model systems has not been reported. The presence of IQ as intermediate/product(s) of the Maillard reaction has previously been suggested and two alternative theoretical pathways have been proposed. In one, the initial reaction starts from the condensation of creatinine with an aldehyde (Jones and Weisburger, 1989). The subsequent conjugated addition of alkylpyridines or -pyrazines should thereby form the IQ compounds. The second proposed reaction route starts from an aldol-type condensation between alkylpyridines or -pyrazines and carbonyl compounds to form vinylpyridines or -pyrazines. Following conjugated addition of the enol form of creatinine to these intermediates, the reaction should thus form the IQ compounds (Skog and Jagerstad, 1990). The two reaction routes are theoretically intriguing, as either of them should be able to provide experimental evidence for the formation of IQ compounds by the addition of selected alkyl- or vinylpyridine, including 2-methylpyridine or 2-vinylpyridine, to their model systems, respectively (Jones and Weisburger, 1989; Skog and Jagerstad, 1990). In the present study, we report for the first time experimental evidence for the formation of IQ from a heated model system in which 2-methylpyridine, acetylformaldehyde and creatinine were heated in diethylene glycol containing 5% water for 1 h at 140°C.

Materials and methods

Chemicals and materials

Glucose-6-phosphate (G-6-P), nicotinamide dinucleotide phosphate (NADP), copper phthalocyanine cellulose (blue cotton) and acetylformaldehdye were purchased from Sigma (St Louis, MO). IQ was obtained from Wako Chemical Co. (Tokyo, Japan). 2-Methylpyridine, diacetyl, and acetaldehyde were purchased from Fluka (Buch, Switzerland). 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline (MeIQ), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) were kindly provided by Dr S.Grivas (Swedish University of Agriculture Sciences, Uppsala, Sweden). 2-Amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP) was a gift kindly supplied by Drs J.S.Felton and M.G.Knize (Biochemical Sciences Division, University of California, Lawrence Livermore National Laboratory, Livermore, CA). Organic solvents and other analytical chemicals were obtained from Merck (Darmstadt, FRG).

Liquid-reflux model

The model was prepared as previously described with slight modifications (Jones and Weisburger, 1989). The three reactants (2-methylpyridine, creatinine and acetylformaldehyde or acetaldehyde or diacetyl), 26 mmol of each, were refluxed in 60 ml of a solution of diethylene glycol containing 5% distilled water (57:3 ml) for 1 h at 140°C.

Sample extraction and purification

Mutagens in the reaction mixtures were extracted through a XAD-2 column (2.0 × 30.0 cm), acid/basic partition (Jones and Weisburger, 1989) and blue cotton (Hayatsu *et al.*, 1983). The blue cotton extracts were used for mutagenicity testing and were further purified by thin layer chromatography (TLC) and HPLC. TLC of blue cotton extracts was carried out by using a glass-plate impregnated with silica gel (Silica gel 60 F-254; Merck) and chloroform:methanol (90:10, v/v) as the solvent system. The chromatograms were cut into 1 cm strips which were individually extracted by the same solvent mixture. The mutagenic activity of each fraction was examined with the Ames test. The TLC active fraction was subjected to HPLC for further purification (Model D-6500 controlled system, L-6200 gradient pump and L-4500 photodiode array detector; Hitachi Co., Tokyo, Japan). The sample was first injected into a semi-preparative Nucleosil column (10 μm particles, 10 × 250 mm) and eluted at a flow rate of 2.5 ml/min with

a gradient of acetonitrile in 10 mM phosphate/sodium hydroxide (pH 7.2) using the following concentrations of acetonitrile: 0–5 min, a linear gradient of 10–25%; 5–10 min, a linear gradient of 25–35%; 10–20 min, a linear gradient of 35–55%; 20–25 min, and a linear gradient of 55–60%. The temperature of the column was ~26°C (room temperature). Fractions were collected at 1 min intervals for mutagenicity testing. The mutagenic fractions were pooled and loaded

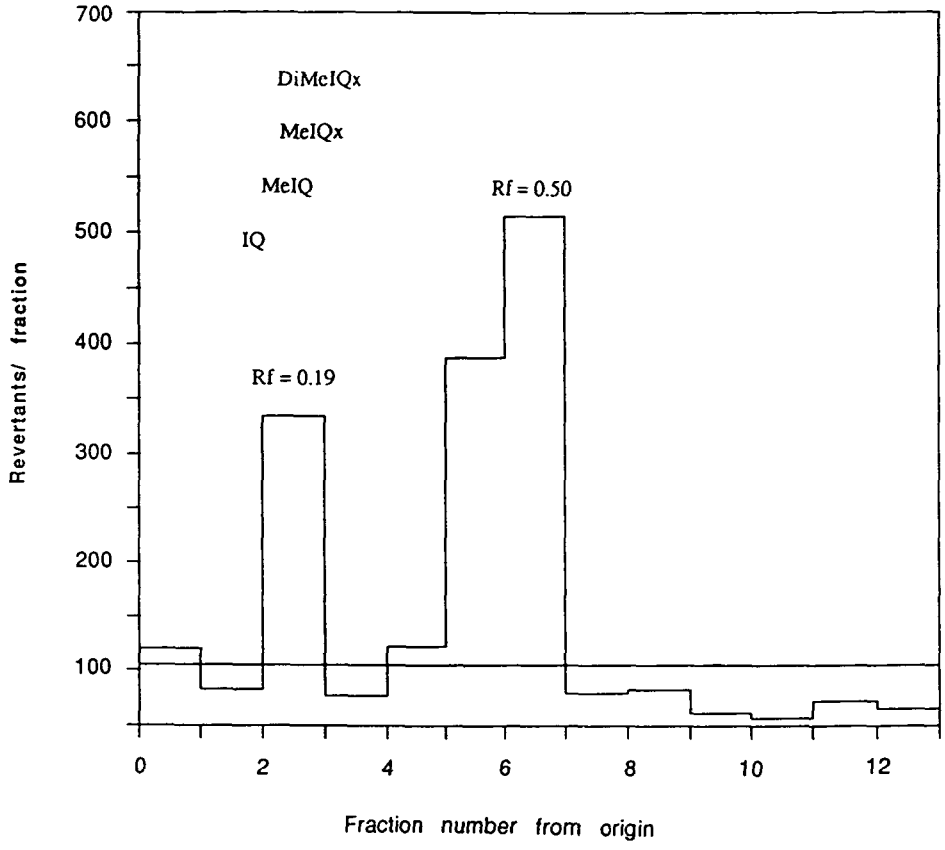

Fig. 1. The mutagenic profile of TLC fractionation from blue cotton extracts of a 2-acetylpyridine/creatinine/aldehyde heated model system. Mutagenicity was tested with *S.typhimurium* TA98 in the presence of S9 mix. The 2-fold background revertant level is indicated with a line.

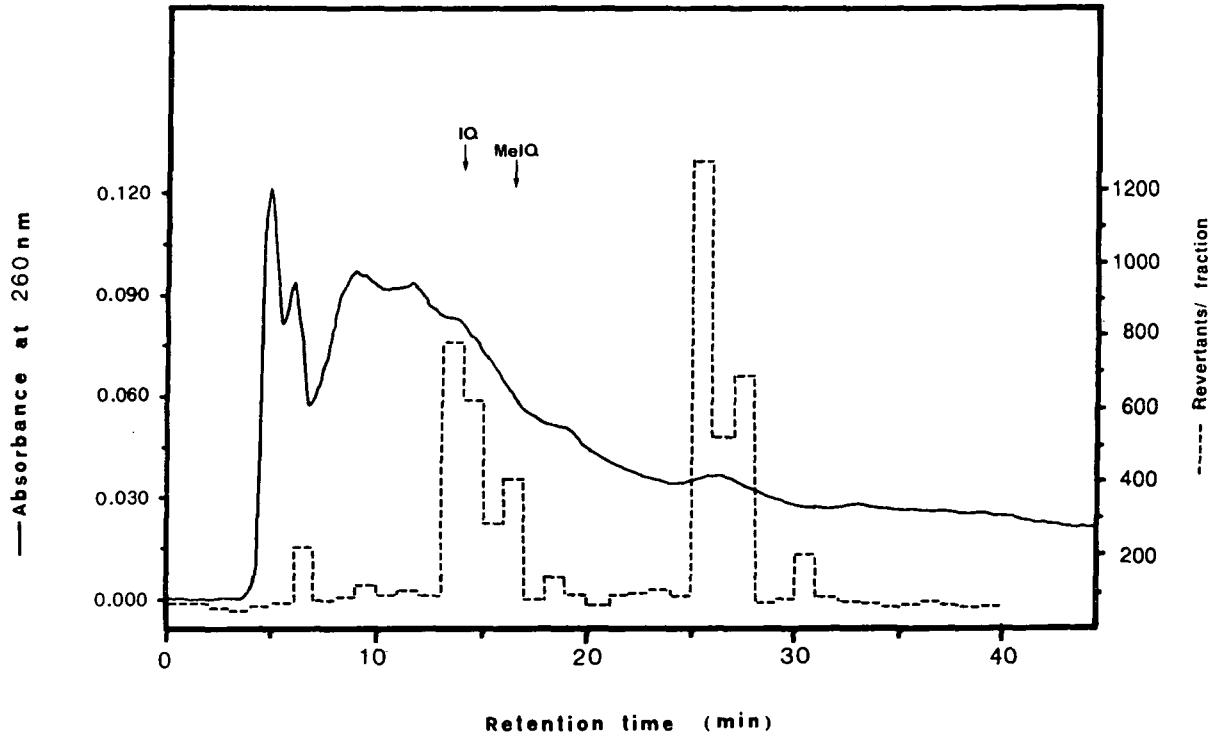

Fig. 2. The elution profile of the extracts of a 2-methylpyridine/creatinine/aldehyde heated model system gathered from semi-preparative HPLC on an ODS column. The mutagenicity of the 1 min fraction was tested with *S.typhimurium* TA98 in the presence of S9 mix.

onto a Nucleosil CN column (5 μm particles, 4.6 × 250 mm). The mobile phase was acetonitrile:water:diethylamine (12:88:0.1, v/v/v, pH 6.0 adjusted with acetic acid) at a flow rate of 1 ml/min. The fractions showing mutagenicity obtained from the second HPLC purification were re-chromatographed under the same conditions. Finally, the fractions showing mutagenicity obtained from the third HPLC purification were injected into a Spherisob pH stable C18 column (5 μm particles, 4.6 × 250 mm). The peak of the sample having the retention time corresponding to the standard mutagenic compounds was monitored with a photodiode array detector to compare the UV spectra with standard IQ. The eluate of the peak was collected for mass spectrometry analysis.

Mass spectrometry identification

Mass spectra were recorded with a JEOL JMS-DX 300 double-focusing instrument using an ionizing energy of 70 eV and an ion-source temperature of 90°C.

Mutagenicity testing

The Ames test was used for the mutagenicity assay (Maron and Ames, 1983). The assay was carried out with *Salmonella typhimurium* TA98 in the presence of S9 mix. The S9 mix contained 25 μl of S9 fraction which was prepared from the livers of Sprague-Dawley rats induced with Aroclor 1254 in a total volume

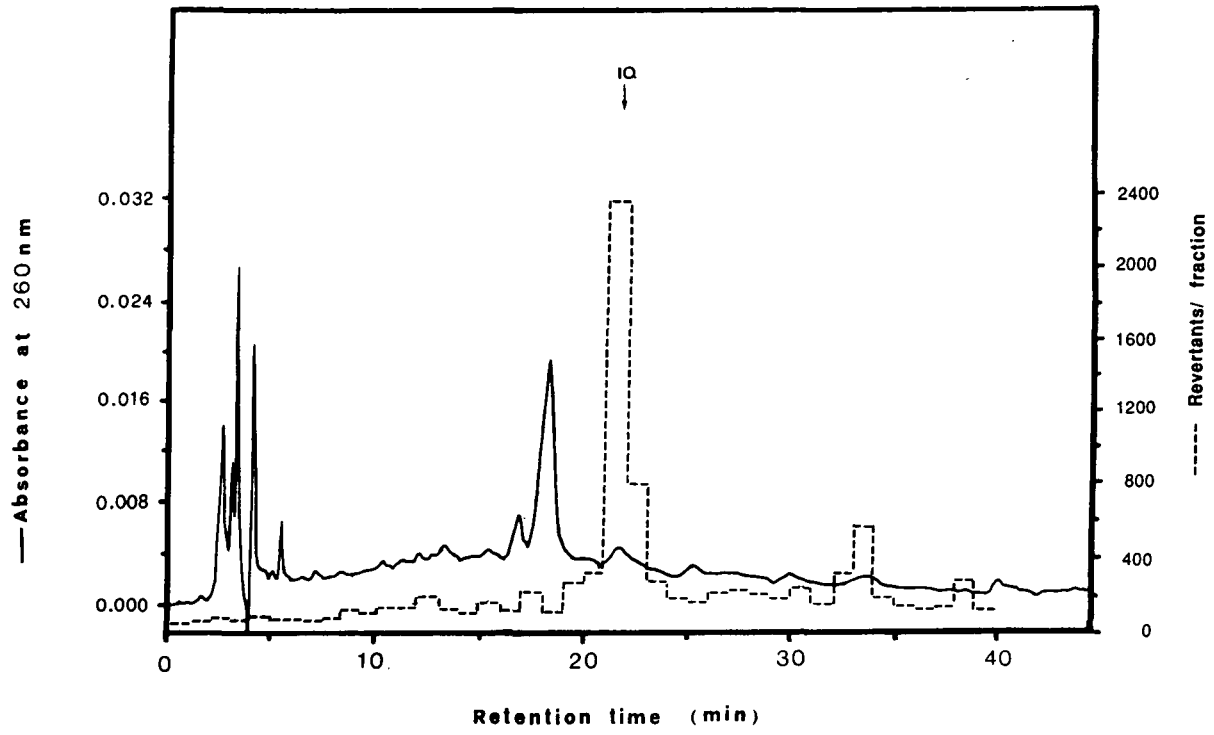

Fig. 3. The elution profile of the extracts of a 2-methylpyridine/creatinine/aldehyde heated model system gathered from HPLC on an analytical Nucleosil 5CN column. The mutagenicity of the 1 min fraction was tested with *S.typhimurium* TA98 in the presence of S9 mix.

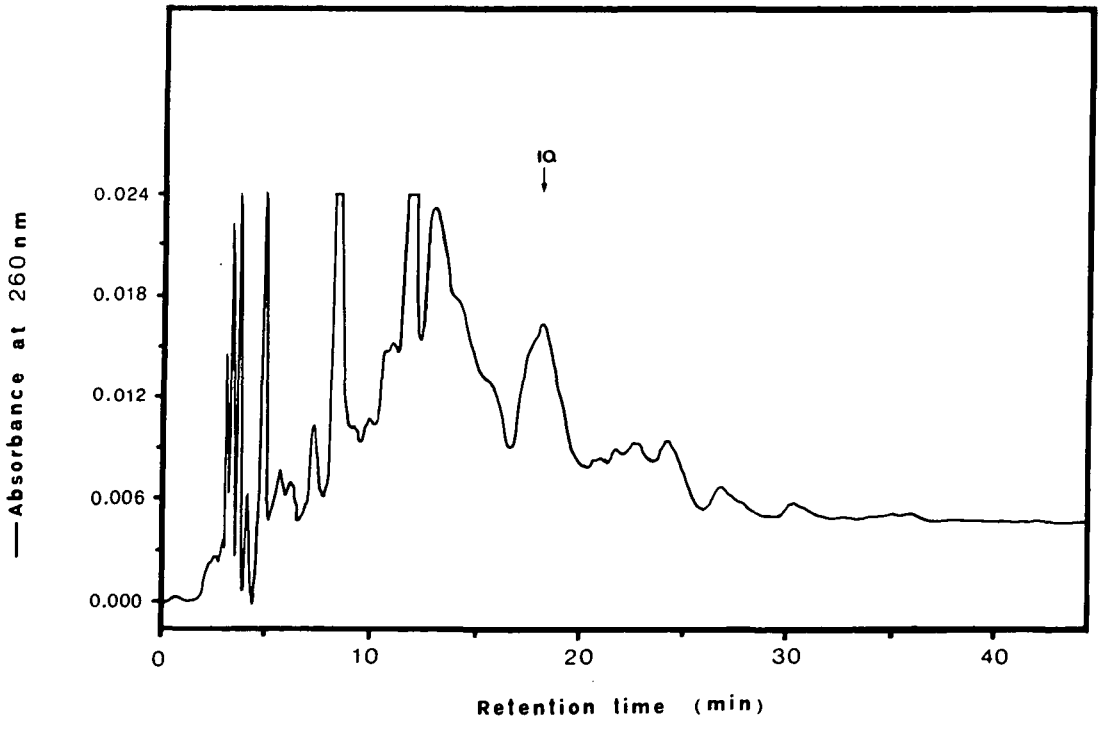

Fig. 4. The elution profile of the extracts of a 2-methylpyridine/creatinine/aldehyde heated model system gathered from the second analytical HPLC on a Nucleosil 5CN column.

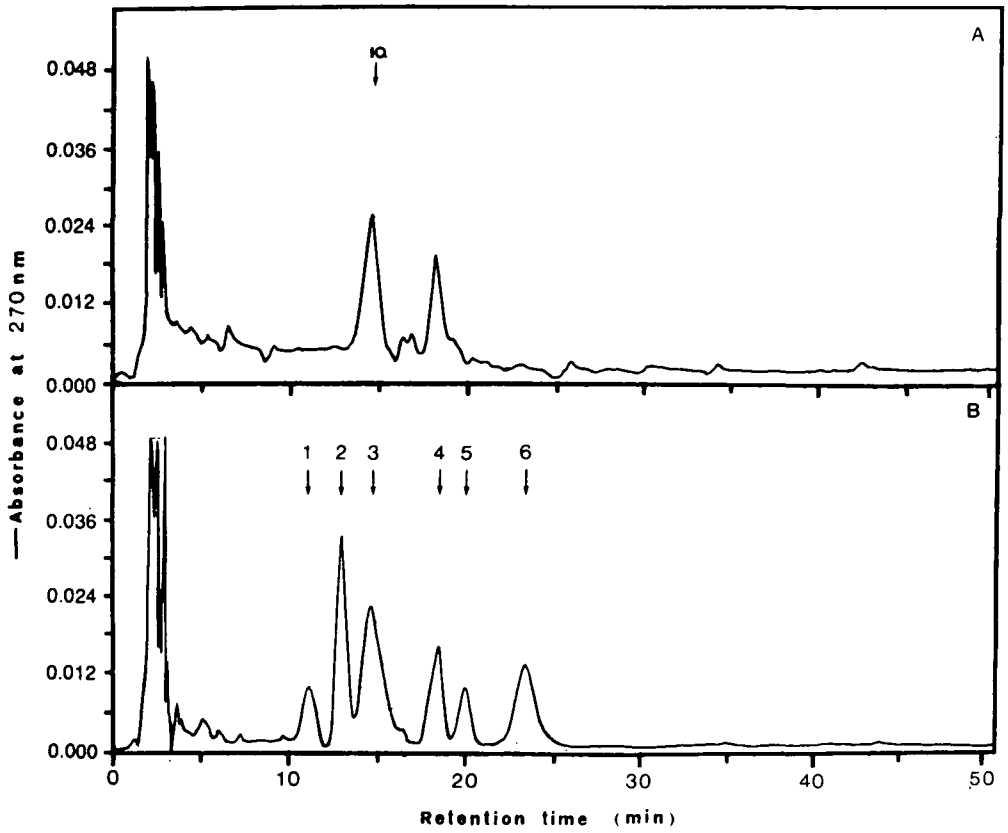

Fig. 5. Elution profiles of the mutagens in a 2-methylpyridine/creatinine/aldehyde heated model system (**A**) and authentic heterocyclic amines (**B**) by HPLC on an analytical Spherisorb 5C18 column. 1, DMIP; 2, MeIQx; 3, IQ; 4, 7,8-DiMeIQx; 5, 4,8-DiMeIQx; 6, MeIQ.

of 500 μl. For tests of mutagenicity, all samples were dissolved in 50 μl dimethyl sulfoxide.

Results and discussion

The mutagenic profile of the 1 cm TLC fraction from extracts of 2-methylpyridine/creatinine/acetylformaldehyde reflux sample was shown to have two mutagenic peaks. The R_f value of the polar fraction (R_f = 0.19) corresponded to that of IQ-type mutagens (Figure 1). Another mutagenic fraction (R_f = 0.50) was significantly more hydrophilic than IQ-type mutagens and may be 2-amino-5-ethylidene-1-methylimidazo-4-one (AEMI), which was obtained from the reflux samples of aldehydes and creatinine (Jones and Weisburger, 1989). The mutagenic fractions were pooled and subjected to HPLC purification. First, the sample was partially purified with a semi-preparative column. The chromatographic and mutagenic profile are shown in Figure 2. The first mutagenic fraction corresponding to the putative IQ mutagen was further purified twice with an analytical CN column. Their HPLC chromatograms and mutagenic profiles are shown in Figures 3 and 4. An increase in the UV absorption (at 260 nm, retention time: 21 min) in the sample, suggests that more mutagenic compounds other than IQ were present in the sample. Finally, IQ in the sample was purified using a Spherisorb C18 column. The peak (Figure 5), with a retention time of 14.8 min, was coincident with authentic IQ, and a comparison of the IQ standard with the mutagenic peak collected from the final HPLC column using a photodiode array detector and mass spectrometry confirmed the presence of IQ (Figures 6 and 7). The amount of IQ yielded in this model system was (as determined by HPLC) 295 ng from 1 g of total reactants. The yield of IQ in our model was lower than that obtained in a creatinine/glycine/fructose model (720 ng/g) by Gravis *et al.* (1986). The low yield is probably due to low temperatures required for the reaction of aldehyde in an aqueous solution (b.p. < 140°C). Models using creatinine/amino acids at temperatures of 180–200°C in dry conditions yield greater amounts of IQ than models using aqueous conditions and lower temperatures (Felton and Knize, 1991).

The effect of various aldeydes on the mutagenicity of the basic extracts of 2-methylpyridine/creatinine/aldehyde reaction mixtures are shown in Table I. The yield of mutagens decreased in the order diacetyl > acetylformaldehyde > acetylaldehyde. Higher yields in mutagens were obtained by adding diacetyl and are probably due to its having a higher boiling point than other aldehydes. Similar observations have been reported by Jones and Weisburger (1989), who also reported on the formation of an IQ-like mutagen, i.e. AEMI, after direct reaction of creatinine and acetylaldehyde or other aldehydes (except for formaldehyde). However, the reaction of an aldehyde with creatinine alone did not yield IQ. Recently, Manabe *et al.* (1992) demonstrated that aldehyde may be a key reactant in the formation of PhIP in a creatinine/L-phenylalanine/aldehyde model system at low temperature (60°C). In these present studies, IQ was produced in a reaction model of 2-methylpyridine/creatinine/acetylformaldehyde. Thus, it can be proposed that pyridines and aldehydes may react together with creatinine to form IQ mutagens through a series of concerted chemical reactions.

In summary, a theoretical pathway for the chemical formation of IQ compounds from pyridine or pyrazine derivatives, an aldehyde and creatine or creatinine has been proposed by Jagerstad *et al.* (1983, 1986). We have used HPLC, mass spectrometry and mutagenicity assays to show that IQ compounds are formed in a reaction model of 2-methylpyridine/creatinine/

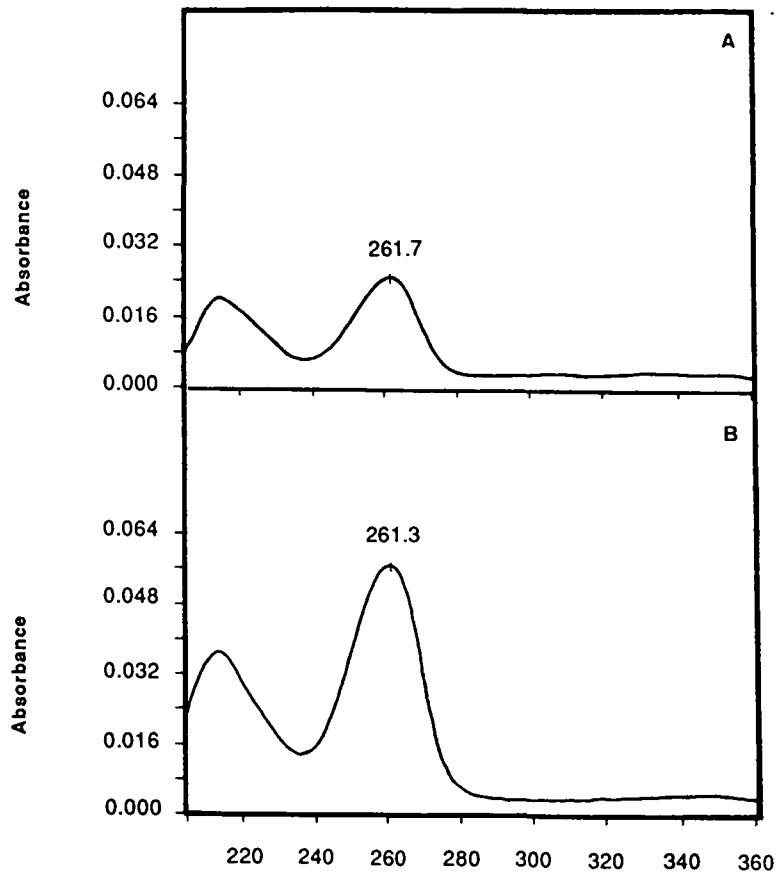

Fig. 6. UV absorbance spectra of purified fraction of a 2-methylpyridine/acetylformaldehyde/creatinine heated model system gathered from HPLC (**A**) and authentic IQ (**B**) monitored with a photodiode array detector.

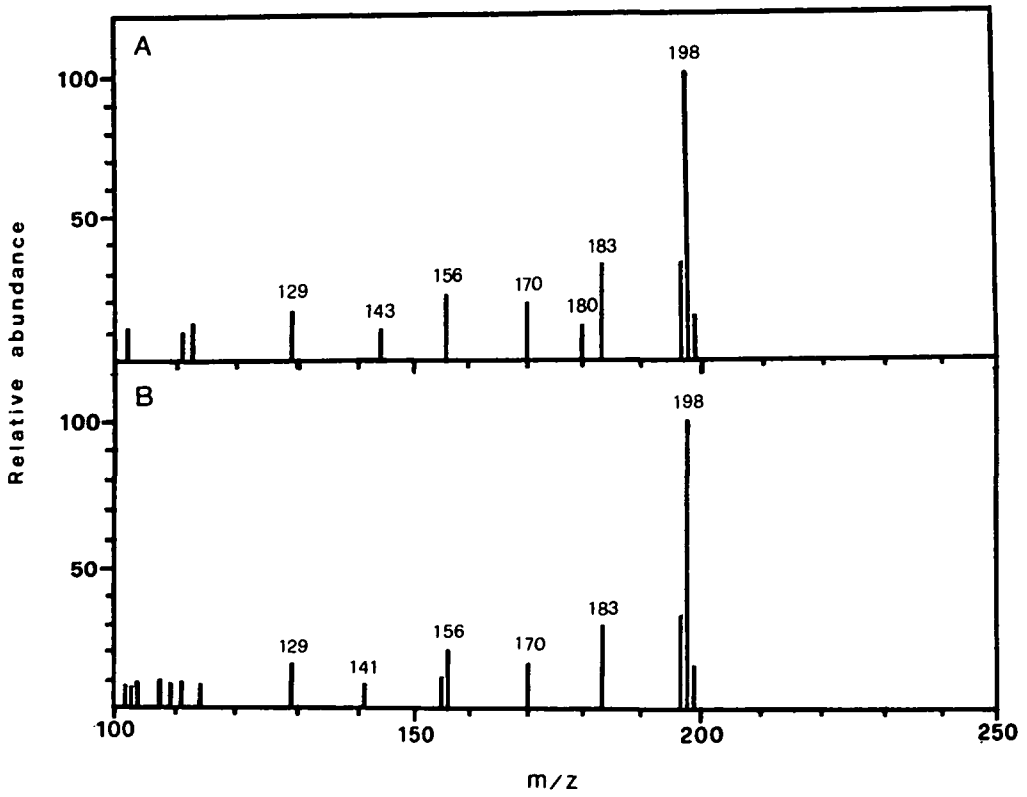

Fig. 7. Mass spectra of authentic IQ (**A**) and the mutagenic fraction of retention time corresponding to IQ from a 2-methylpyridine/acetylformaldehyde/creatinine heated model system (**B**).

Table I. The effect of aldehyde on the mutagenicity of the liquid-reflux model of 2-methylpyridine/creatinine/aldehyde by using *S.typhimurium* TA98 in the presence of S9 mix

Aldehyde	No. of revertants/plate[a]
Acetaldehyde	230 ± 29
Acetylformaldehyde	922 ± 46
Diacetyl	1448 ± 154

[a]One-five hundredth fold of basic extracts of the reaction mixtures were used for the mutagenicity testing per plate.

acetylformaldehyde, which supports the proposed route described by Jagerstad's group. Recent studies showed that carbon atoms from glucose were incorporated into the IQx, MeIQx and 4,8-DiMeIQx (Skog, 1993). In future studies, isotopic labeling will be used to trace the incorporation of aldehyde carbons into IQ compounds, so as to elucidate the role of aldehydes in the formation of IQ compounds.

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