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Inhibition of Aminoimidazoquinoxaline-type and Aminoimidazol-4-one-type Mutagen Formation in Liquid Reflux Models by L-Tryptophan and Other Selected Indoles

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The essential amino acid L-tryptophan (L-Trp) was found to be an effective inhibitor of the development of mutagenicity (Ames test) in liquid-reflux models known to produce identified IQ-type mutagens, such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_X$) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-$DiMeIQ_X$), and in reflux models recently developed in our laboratory that have been found to produce novel IQ-"like" mutagens (aminoimidazol-4-ones), which we have identified as 2-amino-1-methyl-5-propylideneimidazol-4-one (TCP-1), and 2-amino-5-ethylidene-1-methylimidazol-4-one (TCP-2 or ACP). Selected indoles other than L-Trp were also found to be effective inhibitors of mutagen formation in these same reflux models. A mechanism of inhibition of mutagen formation based on the preferential reaction of mutagen precursor aldehydes with the indole-ring nitrogen of these inhibitors, rather than with creatinine, is indicated, and a new "concerted condensation model" for the formation of IQ-type mutagens proposed.

Key words: Mutagens/carcinogens — IQs — Aminoimidazol-4-ones — Inhibition — L-Tryptophan — Indoles

Several specific imidazole-based, heterocyclic amine mutagens (IQs), which are produced during the frying or broiling of meats and fish, and during the heating of appropriate precursor substrates in liquid-reflux model systems, have now been conclusively demonstrated to be multipotential carcinogens in rodents.[1–10] It has been proposed that these potent foodborne mutagens/carcinogens may be the causative agents for the nutritionally-linked human cancers of the breast, colon, prostate, and pancreas, where dietary fat can play a secondary promotional role.[11] Recent reviews have been published by Knudsen,[8] and by Furihata and Matsushima.[12]

While the exact magnitude of human risk inherent in the consumption of IQ-type carcinogens is being deliberated, it seems prudent to develop safe and effective means to inhibit the formation of these carcinogens during the cooking process. We have previously reported the dose-dependent inhibition of the formation of IQ-type mutagens by the amino acid L-tryptophan (L-Trp) in liquid reflux models shown by Jägerstad *et al.*[13] to produce identified IQ-type mutagens, such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_X$), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-$DiMeIQ_X$), etc.[14,15] We have also validated the dose-dependent inhibitory effect of L-Trp during the realistic cooking of meat by applying L-Trp blended into a commercial steak sauce to both surfaces of lean-ground beef patties prior to frying or broiling.[14] In all studies, L-Trp was found to be an effective dose-dependent inhibitor up to 100%.

The present studies were conducted to determine the mechanism of inhibition by L-Trp and other selected indoles, and to provide additional insight into the actual mechanism of formation of the IQ-type mutagens. To these ends, we have utilized simple, 2-component, liquid-reflux model systems, which we recently developed, that we have now demonstrated result in the formation of a new class of low-molecular-weight, imidazole-based, IQ-"like" mutagens (aminomethyl-

Abbreviations: L-Trp, L-tryptophan; TCP-1, 2-amino-1-methyl-5-propylideneimidazol-4-one; TCP-2 (or ACP), 2-amino-5-ethylidene-1-methylimidazol-4-one; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; gluc, D-glucose; Gly, glycine; Cr, creatinine; Thr, threonine.

imidazol-4-ones). These new IQ-like mutagens are produced by heating creatinine with an equimolar quantity of either L-threonine or with acetaldehyde, in a liquid-reflux model of diethylene glycol containing 5% distilled water at 150° for 2 hr. The IQ-like mutagens were identified as: (a) 2-amino-1-methyl-5-propylideneimidazol-4-one (TCP-1) and (b) 2-amino-5-ethylidene-1-methylimidazol-4-one (TCP-2 or ACP).[15]

Materials and Methods

Liquid-reflux Models All substrates were prepared at a concentration of 70m*M* in a total volume of 60 ml of diethylene glycol containing 5% distilled water (57:3 ml), unless otherwise noted. Samples were refluxed at 150° on an electric heating mantle for 2 hr, and aliquots of the crude reflux samples were assayed directly (100 μl/plate) for mutagenicity in the Ames *Salmonella typhimurium* test (TA98+S9 mix).

Creatinine was refluxed with either Thr or acetaldehyde, or D-glucose (gluc) (35m*M*) + glycine (Gly); all were done both with and without the presence of L-Trp. Additionally, the glucose + glycine + creatinine mixture, and the Thr + Cr mixture, were refluxed with and without an equimolar concentration of one of the following selected indoles: L-tryptamine, indole, indole-3-carbinol, L-Trp, N-acetyl-L-tryptophan, or indole-3-carboxaldehyde.

Mutagenicity Assays Ames *Salmonella typhimurium* tester strain TA98 was used in all assays for mutagenicity. The S9 supernatant (40 mg protein/ml) was obtained from liver of Aroclor 1254-treated rats. Of this, 50 μl was used per ml of assay mixture.

The assay was conducted by the addition of 0.1 ml of overnight culture of TA98 bacterium to 0.5 ml of S9 mix (=1 mg protein in assay) plus either 100 μl of crude reflux sample (or its basic extract), or a known concentration of IQ-mutagen reference standard dissolved in diethylene glycol. The mixture was then incubated at 37° for 20 min. Molten top agar was added, and the mixture overlayed onto Vogel-Bonner agar plates. Plates were incubated at 37° for 48 to 72 hr. Assays were conducted in triplicate for each sample, and the results expressed as the average number of revertant colonies (avg.rev.col./plate), as determined with an Artek model 880 automatic colony counter.

Level of significance was defined in the present studies to be 2 times the background (medium) negative control values, as indicated by a superscript [a] placed after the tabulated avg.rev.col./plate value.

Results

L-Trp (70m*M*) inhibited the formation of mutagens in both the Cr (70m*M*)+Thr (70 m*M*) and the Cr (70m*M*)+acetaldehyde (70 m*M*) models (Table I). Thr+Cr produced 1290±73 avg.rev.col./plate without L-Trp, while the presence of L-Trp completely blocked mutagen formation (60±2). In the acetaldehyde + Cr model, 70m*M* L-Trp reduced the mutagenic yield from 920±107 to 170±5, an 88% reduction. L-Trp (70m*M*) lowered the mutagenicity in the glucose (35 m*M*) + Gly (70m*M*) + Cr (70m*M*) model by 80%, 850 vs 205 avg.rev.col./plate (Table II, Experiment 1).

Other selected indoles (70m*M*) displayed various inhibitory effects on mutagen formation, depending upon the specific indole and the reflux model employed (i.e., Thr+Cr, or glucose+Gly+Cr). As shown in Table II, the order of effectiveness (% inhibition) of the selected indoles in the glucose + Gly + Cr model was: indole-3-carboxaldehyde (100%) > L-Trp (80%) > L-tryptamine (65%) > indole (60%) > indole-3-carbinol (35%) > N-acetyl-L-Trp (30%). The order of effectiveness was different for some of the selected indoles in the Thr + Cr model (Table III): indole-3-carboxaldehyde (100%) > L-Trp (90%) = N-acetyl-L-Trp (90%) > indole-3-carbinol (85%) > indole (60%) > L-tryptamine (45%).

Thus, indole-3-carboxaldehyde (100%), L-Trp (80–90%), and indole (60%) were consistently effective in either model, while L-tryptamine was a more effective inhibitor (65%) in the glucose+Gly+Cr reflux model than it was in the Thr+Cr model (45% inhibition). On the other hand, indole-3-carbinol (35%) and N-acetyl-L-Trp (30%) were much less effective in the glucose+Gly+Cr model than they were in the Thr+Cr model, with 85% and 90% inhibition, respectively. The reasons for these discrepancies in effectiveness between the two model systems in regard to L-tryptamine, N-acetyl-L-Trp, and indole-3-carbinol are not understood at this time. Nevertheless, L-Trp and indole-3-carboxaldehyde were consistently found to be highly effective in both reflux models.

In addition, we found in the control experiment (data not shown) that a reflux sample

Table I. Inhibition of IQ-like Mutagenicity by L-Trp in Both the Threonine+Creatinine and Acetaldehyde+Creatinine Models

Model	Ingredient (70m*M*)	Mutagenicity (avg.rev.col./plate) (TA98+S9) Exp. 1	Exp. 2	% Inhibition Exp. 1	Exp. 2
L-Threonine + creatinine	—	1290 ±73[a]	—	—	—
L-Threonine+ creatinine	L-Trp	60 ±2 ($P<0.001$)	—	100	—
Acetaldehyde + creatinine	—	—	930 ±107[a]	—	—
Acetaldehyde + creatinine	L-Trp	—	170 ±5 ($P<0.001$)	—	88
Diethylene glycol-water (negative control)	—	55±2	70±3	—	—
IQ (5 ng/plate) (positive control)[b]	—	610±16[a]	2500±51[a]	—	—

a) Significant level of mutagenicity (2×diethylene glycol-water control)±standard error of the mean (n=3).
b) The values obtained with the positive control varied as a function of the age of the indicator organisms and other factors.
All substrates (70m*M*) were refluxed in diethylene glycol containing 5% distilled water (57:3, diethylene glycol:water) at 150° for 2 hr.

Table II. Inhibition of IQ-type (MeIQx, etc.) Mutagenicity by Various Indoles in the Glucose+Glycine+Creatinine Complete Reflux Model

Model (35m*M*)	(70m*M*)	(70m*M*)	Ingredient (70m*M*)	TA98+S9 (avg.rev.col./plate) Exp. 1	Exp. 2	% Inhibition Exp. 1	Exp. 2
Glucose+glycine+creatinine			Control	850±121[a]	760±45[a]	controls	
〃	〃	〃	+N-acetyl-trp	—	530±39[a]	—	30
〃	〃	〃	+Indole-3-carbinol	570±156[a]	—	35	—
〃	〃	〃	+Indole	380±40[a]	—	60	—
〃	〃	〃	+L-Tryptamine	350±42[a]	—	65	—
〃	〃	〃	+L-Trp	205±7[a]	—	80	—
〃	〃	〃	+Indole-3-carbox-aldehyde	100±14	—	100	—
DEG negative control			—	70±2	50±1	—	—
IQ (5 ng/plate; positive control)			—	1260±128[a]	2320±54[a]	—	—

a) Significant levels of mutagenicity (2 times background control) ± SE (standard error) of the mean (n=3).
All substrates (70m*M*) were refluxed in diethylene glycol containing 5% distilled water at 150° for 2 hr.

Table III. Inhibition of IQ-like (TCP) Mutagenicity by Various Indoles in the Threonine+Creatinine Reflux Model

Model (70m*M*)	Inhibitor (70m*M*)	Experiment 1	Experiment 2	Experiment 3
Thr+creatinine	Control	1200±48[a]	1360±29[a]	1015±123[a]
〃 〃	L-Tryptamine	675±44[a] (45%)[b]	—	—
〃 〃	Indole	530±92[a] (60%)	—	—
〃 〃	Indole-3-carbinol	—	240±3[a] (85%)	—
〃 〃	L-Trp	190±11[a] (90%)	240±7[a] (85%)	135±5[a] (90%)
〃 〃	N-Acetyl-trp	—	—	155±9[a] (90%)
〃 〃	Indole-3-carboxaldehyde	48±3 (100%)	—	—
DEG negative control	—	60±2	55±3	40±5
IQ (5 ng/plate)		830±13[a]	815±27[a]	1600±210[a]

a) Significant levels of mutagenicity (2 times background control) ±SE (standard error) of the mean (n=3).
b) Percent inhibition.
All substrates (70m*M*) were refluxed in diethylene glycol (DEG) containing 5% distilled water at 150° for 2 hr.

of 70m*M* L-Trp alone heated in DEG, for 2 hr at 150°, was not significantly mutagenic, with or without S9 mix, nor did the presence of this sample (100 μl) significantly alter the metabolic activation of IQ standard (5 ng IQ/plate) when applied concomitantly (1100±44 SE without L-Trp sample, versus 1210±484 SE with L-Trp sample).

Discussion

Jägerstad *et al.*[13] have shown that refluxing creatinine with glucose and glycine resulted in the formation of the IQ-type mutagens 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_X$) as 80% of the total mutagenic yield and 2-amino-3, 7, 8-trimethylimidazo [4, 5-*f*]-quinoxaline (7,8-$DiMeIQ_X$) as the remaining 20%, and that refluxing creatinine with fructose and alanine resulted in the formation predominantly of 3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-$DiMeIQ_X$). We reported previously that the essential amino acid L-Trp inhibited the formation of IQ-type mutagenicity in both of these model systems up to 100%, in a dose-dependent fashion.[14] In all such studies, 100% inhibition refers to a decrease in mutagenic yield (avg.rev.col./plate) to below the level of significance (less than 2 times the value found with the background vehicle control). We subsequently validated the inhibitory effect of L-Trp, under realistic cooking conditions, by blending L-Trp at various concentrations into a commercial steak sauce, which was then applied to both sides of a lean ground beef patty prior to frying or broiling.[14] This treatment resulted in a dose-dependent, up to 100%, inhibition of IQ-type mutagenicity in the basic extract of the cooked beef.

In the present studies we have again demonstrated the inhibitory effect of L-Trp in the glucose+Gly+Cr reflux model, but have additionally observed L-Trp to be an effective inhibitor in our two new reflux models: Cr+Thr and Cr+acetaldehyde. We have recently shown that these reflux models produce a new class of imidazole-based, IQ-like mutagens (aminomethylimidazol-4-ones): 2-amino-1-methyl-5-propylideneimidazol-4-one (TCP-1), and 2-amino-5-ethylidene-1-methylimidazol-4-one (TCP-2 or ACP).[15] The HPLC peaks which correspond to these mutagens, isolated from the basic extracts of the respective crude reflux samples, are virtually eliminated from the respective HPLC chromatograms by the addition of equimolar L-Trp prior to refluxing (data not shown). The proposed mechanism of formation of these new IQ-like mutagens is illustrated in Fig. 1.

The Thr + Cr model produces both the TCP-1 and the TCP-2 mutagens, while Cr+acetaldehyde forms only the ACP mutagen, identical to TCP-2. Since L-Trp is an effective inhibitor in the glucose+Gly+Cr model, which produces IQ-type mutagens (i.e. $MeIQ_X$, etc.), as well as in the Thr + Cr and acetaldehyde + Cr models, both of which

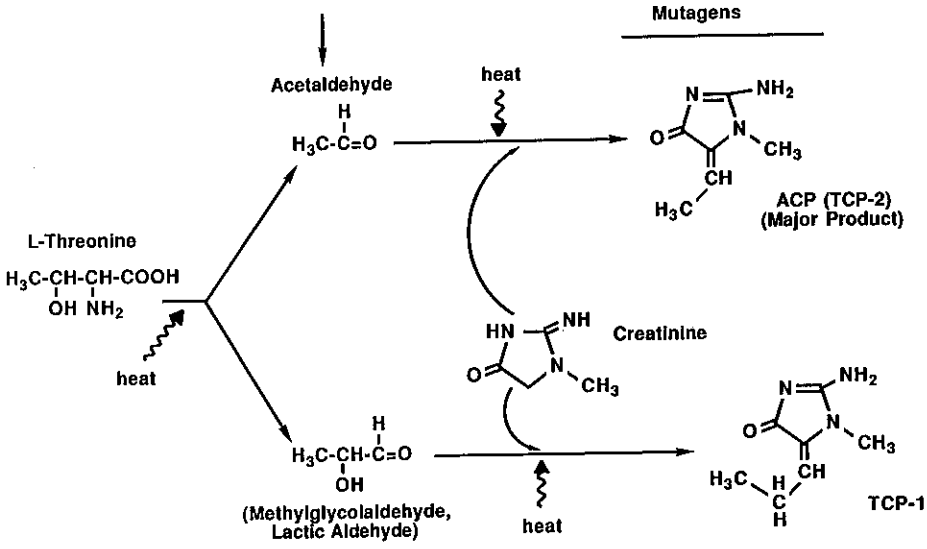

Fig. 1. Mutagen formation pathway derived from results obtained using 70m*M* concentrations of ingredients and refluxing at 150° for 2 hr in diethylene glycol containing 5% distilled water, with identification of resultant aminoimidazol-4-one mutagens by mass spectrometry and proton nuclear magnetic resonance spectrometry.

form IQ-like mutagens (TCP, ACP), the mechanism of formation of these mutagens may share common elements, and L-Trp inhibits the formation of all by the same mechanism. It is of interest to note here that while Thr+Cr produces the two new aminomethylimidazol-4-one mutagens, the addition of glucose to this same reflux model results in the formation of $MeIQ_X$ (25%) and 4,8-$DiMeIQ_X$ (75%), according to Jägerstad *et al.*[13] Thus, additional precursors, which are derived from glucose, alter the resulting mutagenic profile.

The simplest reflux model generating IQ-like mutagenicity is the acetaldehyde + creatinine model, and thus it is proposed that L-Trp inhibits the formation of the 2-amino-5-ethylidene-1-methylimidazol-4-one (ACP) mutagen by competing with creatinine for the acetaldehyde precursor. The mechanism of this competitive inhibition of mutagen formation is presumed to be via the reaction of the indole-ring nitrogen of L-Trp with acetaldehyde. Support for this inhibitory mechanism is gleaned from a recent study by Saito *et al.*[16] in which they show that N-acetyl-L-tryptophan (where the alpha amino group has been rendered unreactive by acetylation) reacts directly via its indole-ring nitrogen with acetaldehyde, glyoxal, or methylglyoxal, under mild heating conditions (50°).[16] Glyoxal and methylglyoxal were found to be more reactive than acetaldehyde with N-acetyl-L-tryptophan, and the reaction products were identified as N-acetyl-1-(2-hydroxy-1-oxoethyl)-L-tryptophan and N-acetyl-1-(1-hydroxy-2-oxopropyl)-L-tryptophan, respectively. Identification of the acetaldehyde plus N-acetyl-L-tryptophan reaction product is currently in progress by their group (Dr. H. Kato, personal communication). Based on this reactivity of indole-ring nitrogen with precursor aldehyde, we refluxed glucose+Gly +Cr, or Thr+Cr with or without equimolar concentrations of selected indole compounds. The Thr+Cr model was selected since it produced a higher level of mutagenicity on an equimolar basis, than did the acetaldehyde +Cr model.

All indoles tested were found to be inhibitory in both model systems, varying in effectiveness of inhibition over a range of 30–100% (Tables II, III). The order of effectiveness was sometimes different in the 2 models. Indole-3-carboxaldehyde yielded 100% inhi-

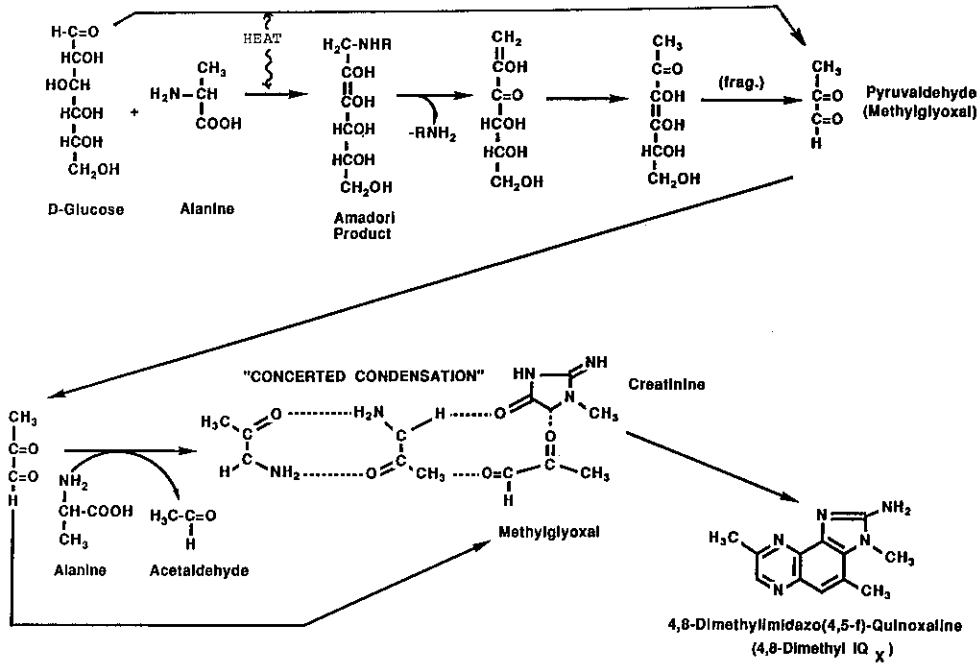

Fig. 2. Hypothetical example of the formation of 4,8-DiMeIQx via the proposed "concerted condensation model" of IQ-type mutagen formation.

bition, L-Trp 80–90%, and indole itself 55% in both models. With the other indoles the effect depended on the model employed. Apparently, the substituent at carbon 3 of indole can modulate the reactivity (inhibitory activity) of the ring nitrogen to some degree in the model systems tested. Nevertheless, all indoles tested had some inhibitory activity.

We are currently seeking to identify the products of the reaction between L-Trp and ^{14}C-acetaldehyde, and to isolate such reaction products from other reflux models where L-Trp has been added, in order to confirm the proposed mechanism of indole inhibition. The fact that L-Trp inhibited the formation of both IQ-like (aminomethylimidazolones) and IQ-type mutagens, presumably by reacting with precursor aldehydes, suggests a more central role for aldehydes in the formation of IQ-type mutagens than has generally been ascribed to them. Thus, we propose a "concerted condensation model" where aldehydes and aminated aldehydes simultaneously react with creatinine in the formation of IQ-type mutagens, as depicted in Fig. 2. By this scheme, dicarbonyls such as methylglyoxal are generated by the rearrangement of Maillard reaction-derived Amadori products. Methylglyoxal may then be aminated by reaction with a free amino acid. These aminated carbonyl compounds may condense with one another, and with unchanged methylglyoxal and creatinine simultaneously (a concerted condensation reaction), to form IQ-type mutagens such as 4,8-DiMeIQx, as illustrated in Fig. 2.

The "concerted condensation model" differs from the conventionally accepted "linear" model of IQ-type mutagen formation, as described by Jägerstad *et al.*[13, 17] The classical, Maillard reaction-based linear model (Fig. 3, pathway 1) suggests that reducing sugars (glucose) are first aminated to glycosylamines (Schiff base) by a Strecker degradation reaction with free amino acids, and then become Amadori products that subsequently rearrange, degrade, fragment, and cyclize to form pyridine and/or more predominantly, pyrazine intermediate precursors of IQ-type

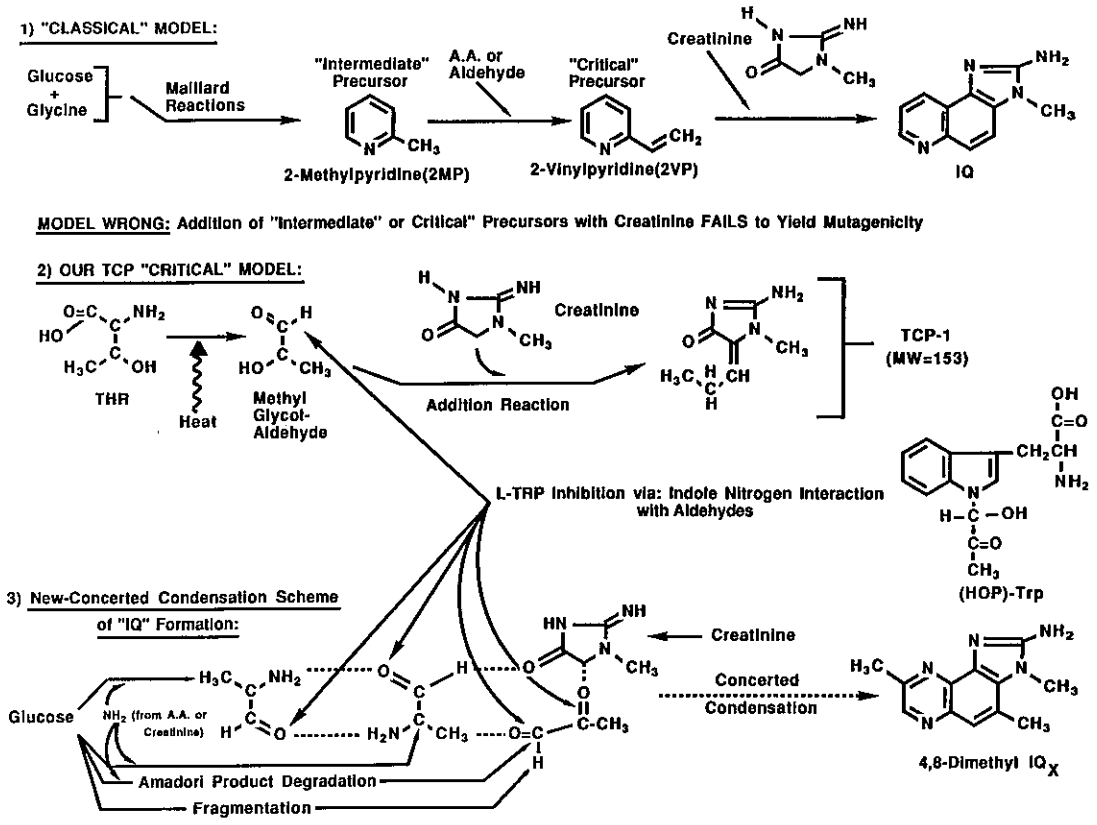

Fig. 3. Comparison of mutagen formation schemes.

mutagens (i.e., 2-methylpyridine). The intermediate precursor then is believed to react with another free amino acid, or an aldehyde, to form an immediate (or "critical") precursor, such as 2-vinylpyridine (2-VP). 2-VP would then react directly with creatinine to form IQ itself. 2-VP is the only commercially available, putative critical precursor for IQ-type mutagen formation, and in previous studies we were unable to produce any mutagenicity in the crude reflux sample of 2-VP and creatinine, even after refluxing 140m*M* concentrations of substrates for 6 hr at 150°.

Thus, the "classical" linear model of IQ-type mutagen formation requires modification, or another mechanism may explain the formation of IQ-type mutagens. We believe that the "concerted condensation model" as described offers such an alternative mechanism.

Indoles, such as L-Trp, inhibit the formation of IQ-type and IQ-like mutagens by reacting with aldehydes (such as methylglyoxal) via their indole-ring nitrogen, thus making such aldehydes unavailable for concerted condensation reactions with other aminated aldehydes and creatinine (Fig. 3; pathways 2 and 3). The type of product thus formed during inhibition would be an L-Trp derivative with the methylglyoxal (aldehyde) adducted to the indole-ring nitrogen, as shown on the right in Fig. 3 and noted as (HOP)-Trp. This compound, if present, would be 1-hydroxy-2-oxopropyl-L-Trp. We have not as yet identified this product from any of our reflux sample basic extracts, but such studies are in progress.

The formation of IQ-type mutagens, derived from the liquid-reflux model glucose + Gly + Cr, and the formation of IQ-like

mutagens, derived from the reflux models Thr + Cr, or acetaldehyde + Cr, was effectively inhibited by the amino acid L-Trp. Other selected indoles were also found to be effective inhibitors in the glucose+Gly+Cr and Thr +Cr models. It is proposed that the mechanism of inhibition is via the reaction of the indole-ring nitrogen of these compounds with crucial precursor aldehydes requisite for mutagen formation, thus rendering the aldehydes unavailable for reaction with creatinine. This mechanism of inhibition of mutagen formation by selected indoles suggests a new "concerted condensation model" for the formation of IQ-type mutagens, rather than the more conventional linear model of their formation. In any case, this model reemphasizes that creatinine is essential[17,18] in the formation of IQ-type and related aminoimidazarenes,[19] as supported by blocking reactions with indoles, including L-Trp, as demonstrated in the present report.

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