Formation of Mutagens in Sugar–Amino Acid Model Systems

Neil E. Spingarn,*[1] Clare T. Garvie-Gould, and Lisa A. Slocum

Mutagens have been shown to form during the cooking of a wide variety of foods. Previous work has linked these mutagens to browning reactions which occur between sugars and amino acids. In this paper the reaction between each amino acid and glucose is shown to have a unique stoichiometry. Analysis of ten analogues of one amino acid, threonine, shows that the structural requirements for mutagen formation are very rigid. A study utilizing starch and protein demonstrates that the complex sugar actually has an inhibiting effect on the mutagen formation of cooked protein. It is concluded that the model systems utilized do not account well for mutagen formation in cooked beef but may be useful for investigating mutagen formation during the cooking of starchy foods.

Browning reactions are responsible for the development of many of the characteristic flavors and aroma of cooked foods. These reactions, which primarily concern the interaction of sugars and amines, lead to the formation of thousands of products belonging to a wide variety of chemical classes (Pokorny, 1980). For example, a recent review cataloged over 160 heterocyclic compounds formed during browning reactions (Shibamoto, 1980). Among such a large group of compounds, it is perhaps not surprising that some of them would be mutagenic.

The mutagenicity of cooked meats was first described by Nagao et al. (1977). This finding has been extensively studied by this and many other groups (Commoner et al., 1978; Felton et al., 1981; Pariza et al., 1979; Rappoport et al., 1979; Spingarn and Weisburger, 1979). Recently, one of the principal mutagens from cooked beef was isolated (Spingarn et al., 1980a) and identified (Kasai et al., 1980b). The compound, 2-amino-3-methylimidazo[4,5-*d*]quinoline, has been synthesized (Kasai et al., 1980a) and is now undergoing assays for carcinogenicity.

Our earlier studies demonstrated that mutagenicity can form in foods even at relatively low (approximately 100 °C) temperature (Spingarn and Weisburger, 1979). This led to studies which found a parallel between browning reactions and mutagen formation in sugar–ammonia model systems (Spingarn and Garvie, 1979). In relating such a model system to meat, the ammonia is presumed to originate from the deamination of amino acids (Koehler et al., 1969). However, amines such as amino acids can also react directly with sugars and sugar breakdown products (i.e., aldehydes and ketones) to form intermediates of varying stability. These intermediates are further degraded and interact to form the myriad products observed (Shibamoto and Bernhard, 1977). The sulfur-containing amine, cysteamine, has been shown to produce mutagens when refluxed with glucose (Mihara and Shibamoto, 1980).

The interactions between sugars and amino acids have been extensively studied due to their importance to the food industry. While most have studied conditions which favor or reduce browning (El'Ode et al., 1966; Rooney et al., 1967; Schroeder et al., 1955), others have studied the basic mechanisms and formation of specific products (Hodge, 1953; Koehler et al., 1969). Still others have focused on the nutritional and physiologic consequences of the decrease in amino acids and the increase in new materials which result from browning (Adrian, 1974).

Early researchers found that model system browning investigations are best carried out at alkaline pH (Hodge, 1953; Ellis, 1959). More recent work has delineated the reasons why this is so. The first step in the Amadori reaction is a Schiff base formation between sugars and amino acids. This reaction is base-catalyzed (Haugaard et al., 1951). The breakdown of carbohydrates into reactive units is also favored under basic conditions (Schroeder et al., 1955). A third reaction pathway, the reaction of heat-produced alcohols with amino acids also increases at high pH (Suyama et al., 1979). While the relevance of high-pH model studies to food has been questioned, most studies have indicated that the same reactions occur at neutral pH, only more slowly. Heat degradation of proteins leads to production of free ammonia (Bjarnason and Carpenter, 1970). This will produce locally high pH levels. Since amino acids are less stable in base than in acid (Adrian, 1974), this would be a self-catalyzing reaction. Taking this into consideration, and in order to make our studies fit in with the extensive literature on model systems, we performed our studies of the interaction of glucose with various amino acids under basic conditions.

MATERIALS AND METHODS

Determination of Stoichiometry. Stoichiometry for the reaction between glucose and amino acids was determined by the method of continuous variation. In each of 11 flasks the ratios of amine to glucose varied, but the sum of the molar concentrations of amine (C_A) and glucose (C_G) remained constant at 2.0 M. The mixtures were adjusted to pH 10.5 with NH_4OH before diluting to their final aqueous volume of 100 mL. After the solutions were refluxed for 3.0 h, the pH of each mixture was readjusted to pH 10–10.5 with NH_4OH and extracted with 3 × 50 ml CH_2Cl_2. The organic layers were combined and evaporated to dryness under vacuum, and the residue was dissolved in 1.0 mL of EtOH. Stoichiometries were determined at least twice and the results were averaged. This extraction method has previously been shown to be efficient (Spingarn and Garvie, 1979). Mutagenicity of each sample was determined as described below.

Study of Threonine Analogues. All analogs were refluxed with glucose at $C_A/(C_A + C_G) = 0.8$. Mixtures were prepared and extracted identically with the continuous variation samples. The reaction with each analog was performed 2–5 times and the results from the mutagenicity determinations were averaged.

Study of Protein–Starch Interactions. A continuous variation study was performed where weight of starch (W_S) and protein (W_P) was held constant instead of molarity.

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595.

[1]Present address: WCTS, 17605 Fabrica Way, Cerritos, CA 90701.

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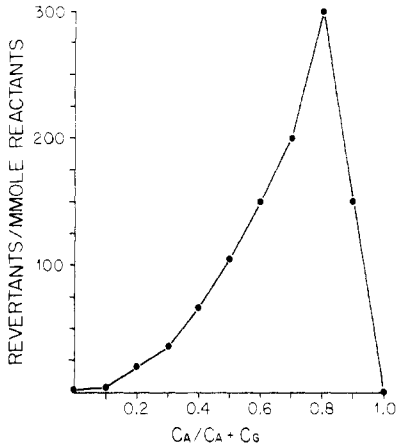


Figure 1. Mutagenicity of serine–glucose reaction products. Ordinate shows nonspontaneous TA98 his$^+$ revertants per mole of total reactants calculated from dose–response curves. The abscissa shows the mole fraction of the amino acid in the sample.

Purified potato starch and purified casein were made into patties consisting of 35 g of water and 50 g of $W_S + W_P$. Duplicate patties were cooked in the absence of oil for 10 min in a frying pan (Silverstone on aluminum) on an electric stove. Pan temperature was 280 °C.

The surface of the patty was scraped off, ground with a mortar and pestle and extracted into acidic water (pH 2 with HCl). The suspension was then saturated with $(NH_4)_2SO_4$ and filtered through glass wool. After the pH was adjusted to 10 with NH_4OH, the samples were again filtered through glass wool. Filtrates were extracted with three aliquots of CH_2Cl_2. The organic layers were combined and evaporated to dryness under vacuum, and the residue was dissolved in 1.0 mL of EtOH. The experiment was repeated twice. Extracts made from the acidified aqueous layers failed to show mutagenicity in preliminary experiments and were not studied further.

Determination of Mutagenicity. *Salmonella typhimurium* strains TA98 and TA100 were kindly supplied by Dr. Bruce N. Ames, University of California, Berkeley, CA. Overnight cultures of bacteria in nutrient broth (Difco 1.5%) were used for the mutagenesis assay. Liver homogenate supernatant (S-9) from rats treated with Arochlor 1254 brand polychlorinated biphenyls was used to make S-9 Mix as described by Ames et al. (1975). Three hundred microliters of S-9 Fraction was employed/mL of S-9 Mix. The standard pour plate assay was utilized for these experiments (Ames et al., 1975). The initial slope of the dose–response curve generated was used for calculations to avoid toxic responses. Aminofluorene was utilized as a positive control for TA98 with S–9 activation.

RESULTS AND DISCUSSION

The interaction between glucose and amino acids was studied by means of the continuous variation technique. This permitted determination of the stoichiometry as well as the intensity of mutagen production from each reaction. Figure 1 shows a typical continuous variation curve for serine. Stoichiometry is plotted against revertants per mole of total reactants. When presented in this manner, the peak of activity represents the approximate optimum stoichiometry. To the left of the peak represents a situation where the amino acid is the limiting reactant; to the right glucose is limiting. With cysteine (Figure 2), increased mole fraction of amino acid led to the formation of a product which was highly toxic to the bacteria. Table I gives the optimal stoichiometry as well as the mutagenicity of the reaction mixture at that stoichiometry. In this table, mole fraction has been converted to stoichiometry (e.g., a mole fraction of 0.5 equals a stoichiometry of 1:1).

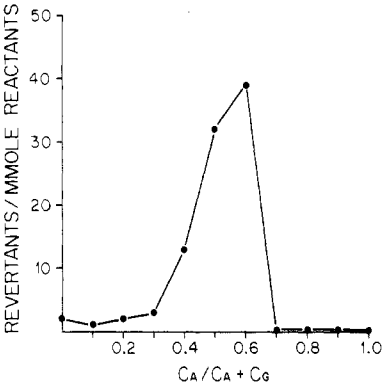


Figure 2. Mutagenicity of cysteine–glucose reaction products. The ordinate and abscissa are as in Figure 1.

Table I. Mutagenicity of Amino Acid-Glucose Reaction Products

amine	optimal stoichiometry[a]	mutagenicity,[b] revertants/mmol: reactants	amino acid	glucose
alanine	9:1	160	190	1300
arginine	1.5:1	30	60	70
cysteine	1:1	50	100	110
glutamic acid	6:1	50	60	440
glycine	6:1	650	810	4200
histidine	1:1	10	20	20
lysine	1.5:1	15	20	30
phenylalanine	4:1	170	250	1000
proline	6:1	120	150	900
serine	3:1	360	500	1500
threonine	3:1	750	1400	3800
tryptophan	9:1	20	20	200
valine	3:1	100	140	430

[a] Molar ratio of amino acid:glucose which resulted in the highest mutagenicity per mole of total reactants based on continuous variation curves. [b] Mutagenicity at the optimal stoichiometry on TA98 with S-9 activation calculated from the slopes of the dose–response curves, expressed per millimole of total reactants, per millimole of amino acid, and per millimole of glucose. Replicate assays were within 20% of each other. The averaged results are presented.

The optimum stoichiometry varies widely, from approximately 1:1 amino acid:glucose to greater than 9:1, the highest ratio tested. These results are highly suggestive that the amino acids do not simply act as sources of nitrogen produced by heat degradation. Rather, each amino acid has its own specific reaction with sugar. Production of mutagenicity does not correlate with rate of reaction with either sugars or hydroxy ketones (Kawashima et al., 1980). Basic amino acids had low stoichiometries (close to 1:1), while neutral and acidic acids had high (>3:1) stoichiometries.

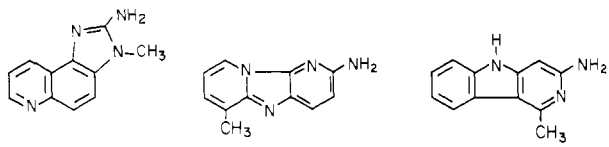
Mutagenicity also divides the amino acids into classes depending on their polarity. The greatest activity comes from the neutral hydroxy amino acids, followed by the neutral amino acids and the least activity results from the polar (acidic and basic) amino acids.

When the amino acids are refluxed in the absence of glucose, mutagenicity also appears although it is much weaker than in the presence of the sugar. The results are presented in Table II. For comparison, the amino acid mutagenicity after pyrolysis is also presented. The mutagenicity after pyrolysis is, however, approximately 1000

Table II. Mutagenicity of Amino Acid Heat Degradation Products

amino acid	mutagenicity reflux,[a] revertant/mmol	pyrolysis,[b] revertants/μmol
alanine	0.0	77
arginine	0.3	310
cysteine	0.5	31
glutamic acid	0.4	235
glycine	0.0	9
histidine	0.0	6
lysine	0.5	742
phenylalanine	4.0	129
proline	0.5	95
serine	0.3	100
threonine	0.1	188
tryptophan	2.0	1696
valine	0.5	210

[a] Reflux of 0.2 mol of amino acid in 100 mL of NH_4OH, pH 10.5, for 3.0 h. Samples were extracted and assayed as described in the text for the continuous variation assay. [b] Data adapted from Matsumoto et al. (1977). Pyrolysis was in an evacuated ampule at 600 °C.



a b c

Figure 3. Structures of the highly potent mutagens isolated from (a) cooked beef and fish (Kasai et al., 1980b), (b) pyrolysis of glutamic acid (Yamamoto et al., 1978), and (c) pyrolysis of tryptophan (Sugimura et al., 1977).

times the activity from reflux. It would appear that the processes are quantitatively and qualitatively different. This is interesting in light of the structural similarity between the mutagen reported in beef (Figure 3a) which can form at low temperatures (Spingarn and Weisburger, 1979) and the mutagens from pyrolysis of glutamic acid and tryptophan (Figure 3b–c). All three also are highly active on the frameshift tester strains TA1538 and TA98 and require S-9 activation. They have much weaker activity on the base-pair tester strains and are inactive in the absence of enzymatic activation.

The model system samples described herein do not have the strain specificity of the cooked meat samples or the amino acid pyrolysates. They contain significant activity on TA 100 with S-9 activation. Thus, the model system studies do not clearly mimic mutagen formation in meats. The mutagen from beef and fish (shown in Figure 3a) is not easily isolatable from model system samples. Chromatographic separations show many bands of activity. A recent study (Spingarn et al., 1981) demonstrates an important role for fat in mutagen formation in fried beef patties. So that the reactants involved in mutagen-forming browning reactions can be more closely duplicated, the fats present in foods will have to be added to future studies in model systems.

For determination of what facet of the structure of amino acids is responsible for production of mutagens after reaction with glucose, 10 analogues of threonine were studied. Table III shows that none of the analogues exceeded the mutagenicity produced by threonine (1) and glucose. Homoserine (2) was lower but still had strong activity. Deaminated analogues 10 and 11 showed extremely low activity, similar to the weakly active amino acids arginine, histidine, and lysine. As long as the amine

Table III. Mutagenicity of Threonine Analogue-Glucose Reaction Products

analogue	mutagenicity, revertants/mmol of glucose
1, $CH_3CH(OH)CH(NH_2)COOH$	3700
2, $CH_2(OH)CH_2CH(NH_2)COOH$	1550
3, $CH_2(NH_2)CH(OH)CH_2COOH$	250
4, $CH_2(OH)CH_2CH_2NH_2$	860
5, $CH_3CH(OH)CH_2NH_2$	680
6, $CH_3CH_2CH(NH_2)COOH$	500
7, $CH_3CH(NH_2)CH_2COOH$	50
8, $CH_2(NH_2)CH_2CH_2COOH$	30
9, $CH_3C(CH_3)(NH_2)COOH$	350
10, $CH_3CH(OH)CH_2COOH$	70
11, $CH_2(OH)CH_2CH_2COOH$	70

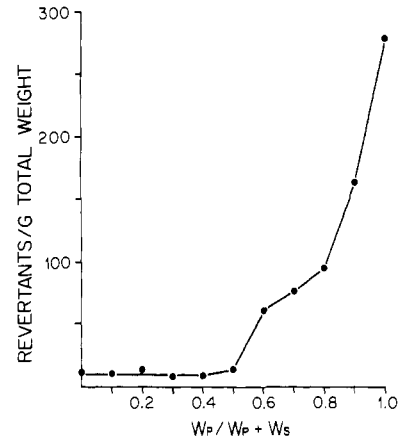


Figure 4. Mutagenicity of cooked protein–starch patties. The ordinate shows nonspontaneous TA98 his^+ revertants per gram of total protein plus starch calculated from dose–response curves. The abscissa shows the weight fraction of protein in the sample.

is α to the carboxyl, as in 6 and 9, some activity remains. When the amine is moved to the β (7) or γ positions (8 and 3), however, activity drops considerably. The carboxyl group, like the hydroxy, is not necessary for activity (4 and 5) but its presence adds a great deal quantitatively. Thus, any change in the configurational structure of threonine results in a decrease in mutagen formation. The most drastic changes are loss or shift of the amine function within the molecule.

In meats, the amino acids would be in the form of protein and the sugars would primarily be as glucose and glycogen. As an intermediate experiment between the model system and food cooking, purified starch and protein were mixed and cooked in a continuous variation experiment ranging from pure starch to pure protein. Figure 4 shows that the mutagenicity of the cooked product continued to rise with increasing ratio of amino acid:glucose with pure protein yielding maximum mutagenicity.

If the only effect of starch was to dilute the mutagenicity from the protein, a linear curve would have been seen between the weight fraction of 0.0 and the maximum at 1.0. The curve found, however, falls entirely below that line, indicating a suppression of mutagen formation by excess starch. The same phenomenon was observed to the left of the optimum mole fraction in the continuous variation curves for the amino acids and glucose. In addition, both the amino acid–glucose and starch–protein mutagens had similar activity on strains TA98 and TA100 with S-9 activation. This lack of strain specificity is also seen in actual cooked foods with high starch content (Spingarn et al., 1980b). It is likely, then, that the sugar–amine model system mimics the browning reactions which occur in

starchy foods but probably not those which occur in meats.

Registry No. 1, 72-19-5; 2, 672-15-1; 3, 352-21-6; 4, 156-87-6; 5, 78-96-6; 6, 80-60-4; 7, 541-48-0; 8, 56-12-2; 9, 62-57-7; 10, 300-85-6; 11, 591-81-1; D-glucose, 50-99-7; starch, 9005-25-8.

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Microbial Metabolism of [^{14}C]Nitroanilines to [^{14}C]Carbon Dioxide

Josef Zeyer and Philip C. Kearney*

A strain of *Pseudomonas* (P6), isolated from soil, grew slowly on *p*-nitroaniline (PNA) as a sole source of carbon. PNA degradation was considerably faster in the presence of yeast extract. A culture grown on 1.5 mM [^{14}C]PNA plus 200 ppm of yeast extract for 8 days released 73% of the initial radioactivity as $^{14}CO_2$ and 16% of the radioactivity was associated with the cells. *o*-Nitroaniline (ONA) and *m*-nitroaniline (MNA) were not degradable as sole sources of carbon. Strain P6, however, was able to degrade [^{14}C]MNA rapidly to $^{14}CO_2$ in the presence of PNA.

Nitroanilines and their derivatives occur in waste water from the dye and pharmaceutical industries and in soils as metabolites from microbial degradation of certain herbicides (Golab et al., 1979; Laanio et al., 1973; Leipzig and Hockenbury, 1979; Stephenson et al., 1979). Literature on the microbial degradation of nitroanilines is very limited. Alexander and Lustigman (1966) incubated 5–10 ppm of *o*-nitroaniline (ONA), *m*-nitroaniline (MNA), and *p*-nitroaniline (PNA), respectively, with soil suspensions and found no significant degradation within 64 days. Young and Affleck (1974) incubated 50 ppm of ONA, MNA, and PNA, respectively, with suspensions of sewage sludge and monitored the biochemical oxygen demand (BOD). The BOD indicated a slow degradation of PNA within 60 days, but no degradation of ONA and MNA was found. Partial degradation of MNA took place within 60 days in the presence of PNA. No direct chemical analysis was performed to verify the degradation of PNA and MNA.

Total degradation of nitroanilines by pure cultures of microorganisms has not been previously reported. Only reduction of the nitro substituent leading to the accumulation of the corresponding diamino derivative or coupling reactions involving the amino substituent were found to occur in pure cultures (Lusby et al., 1980; McCormick et al., 1976).

In contrast to the nitroanilines, microbial degradation of aniline and nitrobenzene derivatives has been well documented. Aniline and aniline derivatives such as *m*-

Pesticide Degradation Laboratory, Environmental Quality Institute, U.S. Department of Agriculture, Beltsville, Maryland 20705.