Factors Affecting the Formation of 4-Methylimidazole in Ammonia-Treated Fodder

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The factors affecting the formation of 4-methylimidazole from reducing sugars and ammonia were studied in model systems consisting either of aqueous solutions of glucose and ammonia or of wet straw, hay, or malted barley and ammonia. Higher sugar and ammonia concentrations, higher temperatures, higher water activity, and longer reaction times generally increased the amounts of 4-methylimidazole formed. However, these different rates of formation occurred at the pH achieved by the added ammonia. The maximum rate of formation, achieved at considerably higher pH, is not affected.

INTRODUCTION

During the 1950s attempts were made to increase the nutritional value of molasses used to feed cattle by adding ammonia to it. On some occasions feeding cattle with the treated molasses resulted in severe intoxications or "bovine bonkers." It was later suspected that these cases were due to 4(5)-methylimidazole (4-MeI) formed from the ammonia and the reducing sugars in the molasses [see Morgan and Edwards (1986a)].

At present, the preservation of straw by treating it with ammonia is a common practice, particularly for straw with a high moisture content, because the addition of ammonia increases the protein value of the roughage. With grain straw there is little risk because it contains virtually no reducing sugar, a prerequisite for the formation of 4-MeI. However, cases of intoxication have been reported after cows have been fed either ammonia-treated grass seed straw (Nielsen et al., 1986) or ammonia-treated grain straw contaminated with weeds (Carlsson, 1987), both of which contain appreciable amounts of reducing sugars. Hay treated with ammonia has also caused intoxication (Fröslie and Bratberg, 1985; Pehrson, 1985; Weiss et al., 1986; Kerr et al., 1987).

It was therefore considered of interest to study the factors that affect the formation of 4-MeI when ammonia is used to improve the quality of roughages. There are several steps in the Maillard-type reaction between sugar and ammonia, and several other substances are formed in addition to 4-MeI (Hough et al., 1952; Wiggins and Wise, 1955). It is therefore possible that 4-MeI is not the only substance responsible for the observed toxicity (Morgan and Edwards, 1986b; Weiss et al., 1986). However, 4-MeI is clearly implicated, and this paper will consider the conditions that affect the formation of 4-MeI.

MATERIALS AND METHODS

The sugar used as substrate for the reaction was supplied either in the form of aqueous solutions of glucose (*the glucose system*) or as finely ground fodder in the form of straw, hay, or malted barley (*the fodder system*). Malted barley was included because it is similar to grain that has started to germinate as a result of poor storage; such grain is often used as fodder. All reactions were carried out in sealed glass vessels placed in a heating cabinet.

With the glucose system, studies were made of the effects of varying the concentrations of the substrates, glucose and ammonia, of sodium chloride (which affects water potential and ion strength), and of poly(ethylene glycol) (which mainly affects water potential); the effects of varying the reaction time, temperature, and pH were also studied.

With the fodder system, the factors studied were the concentration of ammonia, the dry matter content, and the pH.

Chemicals used in the reactions were of reagent grade supplied by Merck. Straw and hay were of local origin, and malted barley was pilsener malt obtained from Söderhamns Malteri.

The concentration of 4-MeI was measured by a reextraction procedure followed by HPLC (Thomsen and Willumsen, 1981).

Glucose System Experiments. *1A. Concentration of Glucose.* The concentration of glucose was varied from 0 to 1.1 M in 0.055 M increments. The solutions also contained 5 M NH_3 and 1.2 M NaCl. The mixtures were allowed to react for 24 h at 50 °C.

1B. Concentration of Ammonia. The concentration of ammonia was varied from 0 to 13 M in 1.3 M increments. The solutions also contained 1.2 M NaCl and 0.4 M glucose. The mixtures were allowed to react for 24 h at 50 °C.

1C. Concentration of NaCl. (a) The concentration of sodium chloride was varied from 0 to 5.1 M in 0.34 M increments. The solutions also contained 0.4 M glucose and 5 M NH_3. The mixtures were allowed to react for 24 h at 50 °C. (b) The concentration of sodium chloride was varied from 0 to 4.4 M in 0.34 M increments. The solution also contained 0.4 M glucose and 1.3 M NH_3. The mixtures were allowed to react for 24 h at 50 °C.

1D. Concentration of Poly(ethylene glycol) (PEG). The amounts of PEG were varied from 0 to 690 g/L. The solutions also contained 0.4 M glucose and 5 M NH_3. The mixtures were allowed to react for 24 h at 50 °C.

1E. Time of Reaction. (a) Solutions containing 5 M NH_3, 0.4 M glucose, and 1.2 M NaCl were allowed to react at 50 °C for 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72, 168, and 192 h. (b) A solution containing 5 M NH_3, 0.4 M glucose, and 4.3 M NaCl was kept at 40 °C and sampled at intervals from 3 days to 3.5 months after it had been prepared.

1F. Temperature. Solutions containing 5 M NH_3, 1.4 M glucose, and 4.3 M NaCl were kept for 24 h at temperatures ranging from 22 to 60 °C with increments of 2 °C.

1G. pH. (a) Solutions containing 5 M NH_3, 0.4 M glucose, and 1.2 M NaCl were adjusted to pH 5–13 with increments of 1 by the addition of HCl or NaOH. The samples were allowed to react at 50 °C for 24 h. (b) Solutions containing 5 M NH_3, 0.4 M glucose, and 1.2 M NaCl were adjusted to pH 11.2–13.4 with increments of 0.2 by addition of HCl or NaOH. The samples were allowed to react at 50 °C for 24 h. (c) Solutions containing 5 M NH_3, 0.4 M glucose, and 4.3 M NaCl were adjusted to pH 11.2–13.4 with increments of 0.2 by addition of HCl or NaOH. The samples were allowed to react at 50 °C for 24 h. (d) Solutions containing 5 M NH_3, 0.4 M glucose, and 1.2 M NaCl were adjusted to pH 10.4–13.4 with increments of 0.2 by addition of HCl or NaOH. The samples were allowed to react at 65 °C for 24 h. (e) Solutions containing 5 M NH_3, 0.4 M glucose, and 1.2 M NaCl were adjusted to pH 10.4–13.4 with increments of 0.2 by addition of HCl or NaOH. The samples were allowed to react at 65 °C for 72 h.

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Table I. Summary of Results Not Presented in Graphs

expt	variable	formation of 4-MeI
1A	glucose concn	asymptotically approached max of 27 mM at approx 0.4 M glucose
1B	ammonia concn	asymptotically approached max of 27 mM at approx 6 M NH_3
1C	NaCl concn	optimum between 1 and 2 M NaCl. (With 5 M ammonia, 4-MeI increased from 17 mM to an optimum of 25 mM and then declined to 20 mM at 4.5 M NaCl. With 1.3 M ammonia, the increase was from 9 to 13 mM and the decline to 10 mM, at the same concentrations of NaCl.)
1D	PEG	linear decline from 27 mM at 0 g/L PEG to 15 mM at 690 g/L PEG
1Ea	time, h	asymptotically approached max of 50 mM after approx 120 h
1Eb	time, days	asymptotically approached max of 45 mM after approx 40 days
1Ga	pH	at pH 5–9, less than 0.4 mM; at pH 10, 1 mM rising to 70 mM at pH 12

Fodder System Experiments. *2A. Amount of Ammonia.* To 10-g aliquots of fodder was added 4 mL of aqueous ammonia with concentrations ranging from 3% to 25%. The samples were allowed to react at 65 °C for 120 h.

2B. Dry Matter Content. (a) To 10 g of straw, 10 g of hay, or 20 g of malted barley were added 3 mL of 25% aqueous ammonia and from 0 to 10.0 mL of water in 0.5-mL increments. The samples were allowed to react for 120 h at 65 °C. (b) To 10 g of fodder were added 6 mL of 25% aqueous ammonia and from 0 to 10.0 mL of water in 0.5-mL increments. The samples were allowed to react for 120 h at 65 °C.

2C. pH. To 10 g of straw, 10 g of hay, or 20 g of malted barley was added 3 mL of 25% aqueous ammonia and (1) 2 mL of 4 M HCl, (2) 1.5 mL of 4 M HCl and 0.5 mL of 4 M NaCl, (3) 1 mL of 4 M HCl and 1 mL of 4 M NaCl, (4) 0.5 mL of 4 M HCl and 1.5 mL of 4 M NaCl, (5) 2 mL of 4 M NaCl, (6) 0.5 mL of 4 M NaOH and 1.5 mL of M NaCl, (7) 1 mL of 4 M NaOH and 1 mL of 4 M NaCl, (8) 1.5 mL of 4 M NaOH and 0.5 mL of 4 M NaCl, or (9) 2 mL of 4 M NaOH. The samples were allowed to react for 72 h at 65 °C.

RESULTS

Effects of Substrate Concentration. Increasing the concentration of either glucose (expt 1A) or ammonia (expt 1B) yielded increased concentrations of 4-MeI (Table I). When fodder was used as a source for sugar, increasing the amount of ammonia also increased the amounts of 4-MeI formed (expt 2A; Figure 1). The straw yielded the smallest amounts of 4-MeI, whereas malted barley yielded the largest amounts. At the highest concentrations of ammonia there was a tendency for the amount of 4-MeI to decrease, particularly with the hay.

Effects of Water Potential, Ionic Strength, and Dry Matter Content. When the concentration of sodium chloride was varied in the glucose system, the formation of 4-MeI reached an optimum at 1–2 M (expt 1C, Table I). When PEG was used to change water potential (expt 1D), there was a steady decline in the amount of 4-MeI formed, from 27 mM with no PEG to 15 mM with 690 g of PEG/L.

In the experiments with fodder there was a decrease in the rate of formation of 4-MeI with increasing dry matter content (expt 2B; Figure 2). At the lower concentration of ammonia (expt 2Ba), however, the amount of 4-MeI formed in malted barley increased slightly.

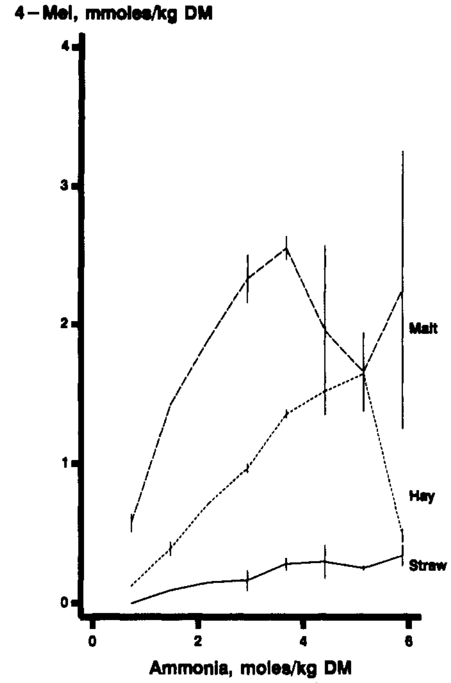

Figure 1. Formation of 4-MeI in the fodder system as a function of the concentration of ammonia.

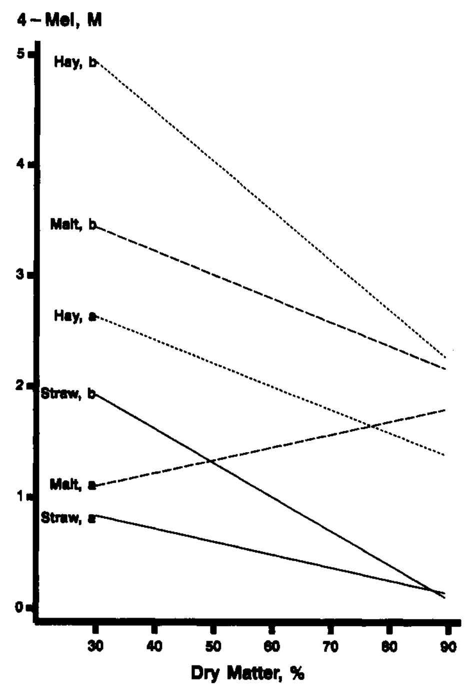

Figure 2. Formation of 4-MeI in the fodder system as a function of dry matter content. The slopes of all the regression lines significantly differ from zero ($p < 0.01$).

Effects of Temperature. The rate of formation of 4-MeI increased approximately exponentially with temperature (Figure 3); an increase in temperature from 40 to 60 °C increased the formation of 4-MeI by a factor of almost 10. At temperatures below 30 °C very little 4-MeI was formed within 24 h.

Effects of Reaction Time. At 50 °C the concentration of 4-MeI did not reach a maximum until after 3 days (expt 1Ea, Table I). At 40 °C (expt 1Eb) the reaction continued for a month, although the rate of the increase in the concentration of 4-MeI decreased with time.

Effects of pH. Increases in pH increased the rate of formation of 4-MeI exponentially (Table I; Figure 4). In the glucose system very little 4-MeI was found below a pH

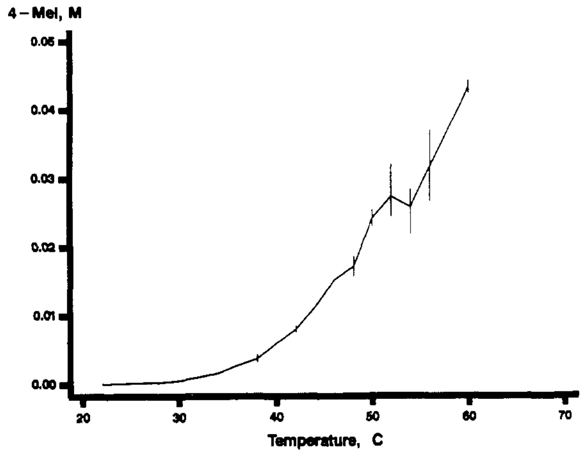

Figure 3. Formation of 4-MeI in the glucose system as a function of temperature.

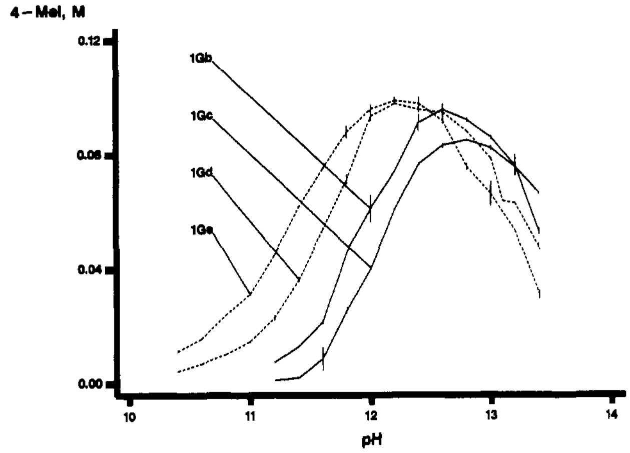

Figure 4. Formation of 4-MeI in the glucose system as a function of pH.

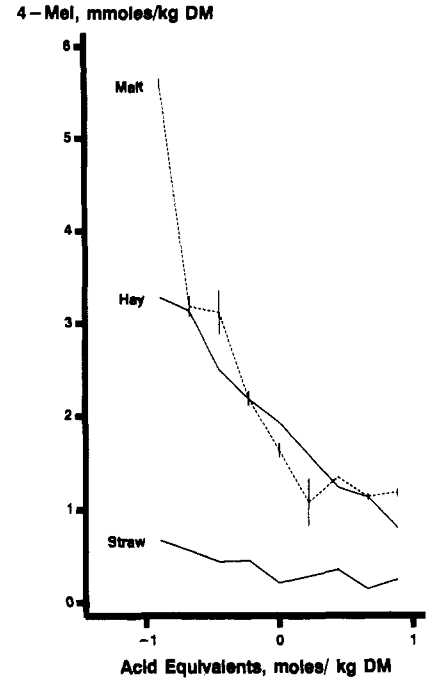

Figure 5. Formation of 4-MeI in the fodder system as a function of acid equivalents added. Additions of base are represented as negative equivalents of acid.

of 10. At higher pH the concentration of 4-MeI increased, with an optimum at about pH 12–12.5, depending on reaction conditions. Higher temperatures and longer reaction times tended to push the optimum pH to lower values (Figure 4). An increase in the concentration of sodium chloride from 1.2 to 4.3 M pushed the optimum toward a higher pH.

In the fodder system, the amount of 4-MeI formed decreased as the amount of acid equivalents added was increased (expt 2C; Figure 5). The pH achieved ranged approximately from 9 to 10.5.

DISCUSSION

In general, the results of these experiments agree with those of Nielsen et al. (1986) and Perdok and Leng (1987). Higher concentrations of sugar and ammonia, higher temperatures, higher water activity, and longer reaction times resulted in more 4-MeI being formed. However, these differences in the rate of formation of 4-MeI occurred at whatever pH was produced by the treatment with ammonia. The shape of the curve describing the formation of 4-MeI as a function of pH remained nearly constant, and the curve was displaced along the pH axis (Figure 4), without affecting the maximum rate of formation of 4-MeI.

The optima which occurred in the glucose system when the pH was changed and when the concentration of sodium chloride was changed indicate that these factors affect the reaction at several points. The protonation of ammonia to ammonium is probably the factor that limits the rate of formation of 4-MeI at pH levels below the pK_a of ammonium. The effect of increasing the pH to 3 pH units above the pK_a must be due to effects on the reaction mechanisms. The optimum which occurred between 1 and 2 M sodium chloride may indicate that while decreasing water activity inhibits the formation of 4-MeI, the corresponding increase in ionic strength has the opposite effect.

It is evident that the use of ammonia for improving the quality of fodders should be limited to fodders which contain little or no reducing sugar. The attempt to improve hay or germinated grain involves some risk because they are comparatively rich in reducing sugars. The amount of ammonia used can affect the rate of formation of 4-MeI in other ways than by the direct effect of concentration, because it also affects the pH and temperature of the treated fodder. The ammonia is usually applied as gaseous ammonia, which dissolves rapidly in the moisture in the fodder with the production of heat. Temperatures of 70 °C may be reached and, because the outer layers of a bale of straw or hay provide efficient insulation, the temperature inside a bale may be raised for several weeks after treatment (Atwall et al., 1986). The increased reaction time and temperature will tend to reduce the optimal pH for the formation of 4-MeI, and it is possible that 4-MeI may be formed at a considerably lower pH, given reaction times lasting for several weeks.

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LITERATURE CITED

Atwal, A. S.; Heslop, L. C.; Lievers, K. Effectiveness of anhydrous ammonia as a preservative for high-moisture alfalfa hay in large round bales. *Can. J. Anim. Sci.* **1986**, *66*, 743–753.

Carlsson, J. *Sv. Veterinärtidning.* **1987**, *39*, 825–828.

Fröslie, A.; Bratberg, B. *Nor. Veterinärtidsskr.* **1988**, *100*, 213–214.

Hough, L.; Jones, J. K. N.; Richards, E. L. The reaction of amino compounds with sugars. Part I. The action of ammonia on D-glucose. *J. Chem. Soc.* **1952**, 3854–3857.

Kerr, L. A.; Groce, A. W.; Kersting, K. W. Ammoniated forage toxicosis in calves. *J. Am. Vet. Med. Assoc.* **1987**, *191*, 551–552.

Morgan, S.; Edwards, W. C. Bovine bonkers: new terminology for an old problem. A review of toxicity problems associated with ammoniated feeds. *Vet. Hum. Toxicol.* **1986a**, *28*, 16–18.

Morgan, S.; Edwards, W. C. Pilot studies in cattle and mice to determine the presence of 4-methylimidazole in milk after oral ingestion. *Vet. Hum. Toxicol.* **1986b**, *28*, 240–242.

Nielsen, T. K.; Wolstrup, C.; Witt, N.; Friis Kristensen, V.; Kraul, I. 4-methyl-imidazol in ammoniated roughage. Preliminary investigations. Presented at the 6th International Conference on Production Disease in Farm Animals, Belfast, 1986.

Pehrson, B. *Sv. Veterinärtidning.* **1985**, *37*, 537–541.

Perdok, H. B.; Leng, R. A. Hyperexcitability in cattle fed ammoniated roughages. *Anim. Feed Sci. Technol.* **1987**, *17*, 121–143.

Thomsen, M.; Willumsen, D. Quatitative ion-pair extraction of 4(5)-methylimidazole from caramel colour and its determination by reversed-phase ion-pair liquid chromatography. *J. Chromatogr.* **1981**, *211*, 213–221.

Weiss, W. P.; Conrad, H. R.; Martin, C. M.; Cross, R. F.; Shockey, W. L. Etiology of ammoniated hay toxicosis. *J. Anim. Sci.* **1986**, *63*, 525–532.

Wiggins, L. F.; Wise, W. S. Some preliminary observations on the nature of ammoniated molasses. *Chem. Ind.* **1955**, *23*, 656–657.

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Registry No. 4-MeI, 822-36-6; NH_3, 7664-41-7; glucose, 50-99-7.