COMMUNICATIONS

Nitrogen-15 Nuclear Magnetic Resonance of the Amorphous Polymer of Lysine-Formaldehyde-Urea

Nitrogen-15 NMR was used to study the reaction site of the α- and ϵ-nitrogen of lysine in a lysine–formaldehyde–urea polymer. Those results confirm earlier ^{13}C NMR which suggested that only the ϵ-nitrogen is bound to a carbon derived from formaldehyde and linked to urea. The chemical shift of the ϵ-nitrogen changed from −42.16 ppm for lysine hydrochloride to −35.49 ppm for the lysine–formaldehyde–urea polymer while the α-nitrogen remained essentially unchanged (−34.63 to −34.41 ppm, respectively). This shift for the α-nitrogen is within the resolution of the instrument (i.e., less than one data point). Drastic differences in the NOE which can occurr in ^{15}N spectra make quantitative studies difficult. However, the absence of a discernible resonance for unincorporated ϵ-nitrogen in the polymer spectrum supports earlier findings that the incorporation of the lysine in the polymer is virtually complete (>85%). The resonances downfield at about 5 and 19 ppm are probably due to mono- and disubstituted urea nitrogen.

Ruminants do not compete with man for food per se. The ruminant, because of the bacteria and protozoa in the ruminoreticulum, can utilize energy from fibrous feedstuffs and digest the microbial protein produced. The amount of protein synthesized may not be sufficient for optimal growth or milk production (Hungate, 1965). The quality of the microbial protein, i.e., a deficiency in one or more essential amino acids, may be limiting production. Studies by Nimrick et al. (1970a,b) showed methionine, lysine, and threonine also deficient in microbial protein for lambs. Schwab et al. (1976) showed lysine and methionine to be the first and second limiting, respectively, or colimiting amino acids for dairy cattle. The infusion of essential amino acids into a ruminant's abomasum has improved the performance of growing cattle (Chalupa et al., 1973; Boila and Deubin, 1972; Fenderson and Bergen, 1975). However, the supplementation of ruminant diets with free amino acid has been shown to be impractical because the rumen microorganisms rapidly degrade the amino acids (Broderick, 1975). A number of attempts to protect the amino acids from degradation by rumen microorganisms on the way to the abomasum where absorption can occur have been made (Amos et al., 1974; Langer et al., 1975; Richardson et al., 1976).

A recent report by Barton et al. (1979) described the structure of an amorphous polymer of lysine–formaldehyde–urea. The polymer was synthesized so that lysine would be protected from deamination by rumen microorganisms and thus be available for absorption in the animal's true stomach (abomasum). In this study, ^{13}C NMR was used to characterize the polymer, obtain quantitative data as to the amount of free and bound lysine, and observe its hydrolysis under the conditions of the abomasum (i.e., 39 °C with pH <2.5). The structure of the polymer was determined to be a matrix with methylene bridges, derived from formaldehyde, connecting the ϵ-amino group. These structural assignments were made on the basis of shifts in the ϵ-carbon of lysine when the ϵ-amine was bound (44.6 ppm) or free (39.6 ppm) and on the formaldehyde methylene bridges shown by ^{13}C enrichment.

The ^{13}C spectra in the study of Barton et al. (1979), while simpler than ^{1}H spectra, were still quite complicated. For further simplification of the spectra and observation of the reaction site itself, the natural abundance ^{15}N NMR spectra were obtained. These spectra contained resonances for the α- and ϵ-amino groups on lysine and for the nitrogens from urea in their various magnetic environments due to reaction with formaldehyde.

Table I. Chemical Shifts of Lysine Nitrogen Referenced to Urea[a]

compd	chemical shift, ppm	
	α-N	ϵ-N
lysine hydrochloride	−34.63	−42.16
lysine–formaldehyde	−34.63	−41.51
lysine–formaldehyde–urea	−34.41	−35.49

[a] The urea chemical shift (σ) is 0 ppm at 347.66 Hz.

MATERIALS AND METHODS

All samples were prepared as described by Barton et al. (1979). Samples were run on a JEOL FX-90Q at 9.04 MHz. Line positions were referenced to ^{15}N-enriched urea with a D_2O capillary lock. Spectra were taken with a 45° pulse angle of 17 μs pulse width or 60° pulse angle for a pulse width of 23 μs. The pulse repetition rate was routinely 10 s. All spectra were taken at room temperature ($\simeq$22 °C). The spectrometer observation frequency was 9.04 MHz. The spectrometer was locked on deuterium at 13.8 MHz on the upper-side band. Therefore, in all spectra, the field (H_0) increases to the right. Proton decoupling irradiation frequency was 90.0 MHz. Spectral width was 8 kHz, with 8K data points.

RESULTS AND DISCUSSION

Chemical shifts for the α- and ϵ-nitrogen of lysine are given in Table I. Since only the ϵ-nitrogen shifts, there appears to be no incorporation of the α-nitrogen in the lysine–formaldehyde–urea polymer. These shifts are referenced to urea set at 0.0 ppm. The chemical shift for urea on the screening constant scale is 299 ppm upfield from that for nitromethane in nitric acid (Witanowski et al., 1973). The calculated chemical shifts for the α- and ϵ-nitrogen of lysine monohydrochloride are therefore 333.6 and 341.1 ppm, respectively. These values compare fa-

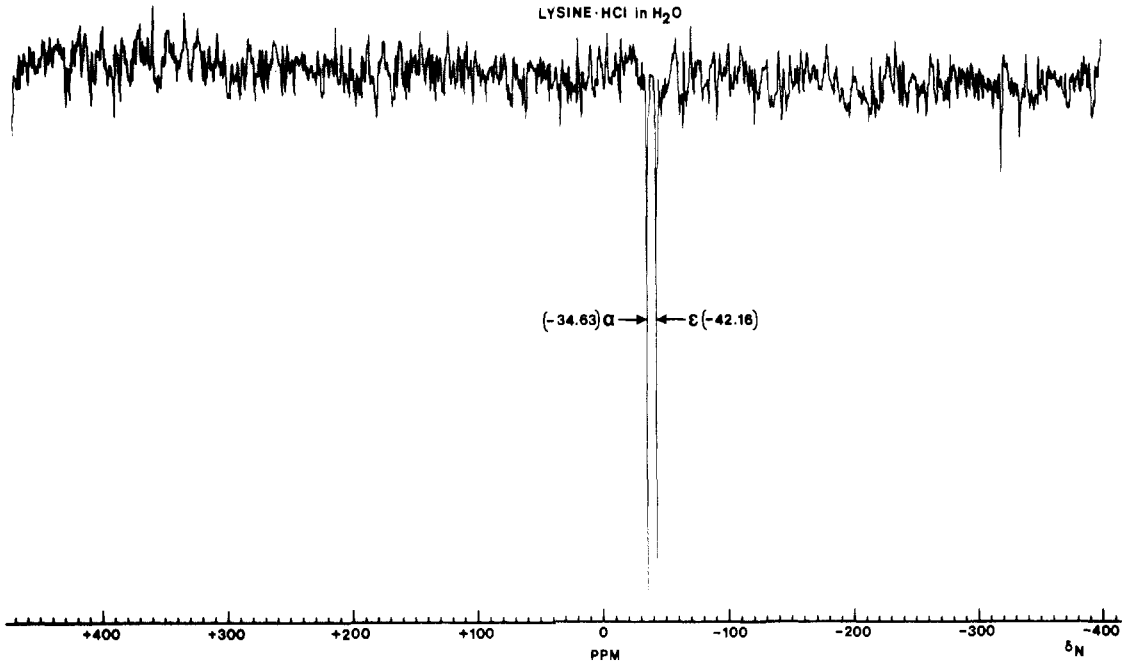

Figure 1. The 7200-Hz ^{15}N spectrum of lysine hydrochloride in water. The two lines are the α- and ϵ-nitrogens of lysine slightly upfield at -34.63 and -42.16 ppm from urea at 0.0 ppm.

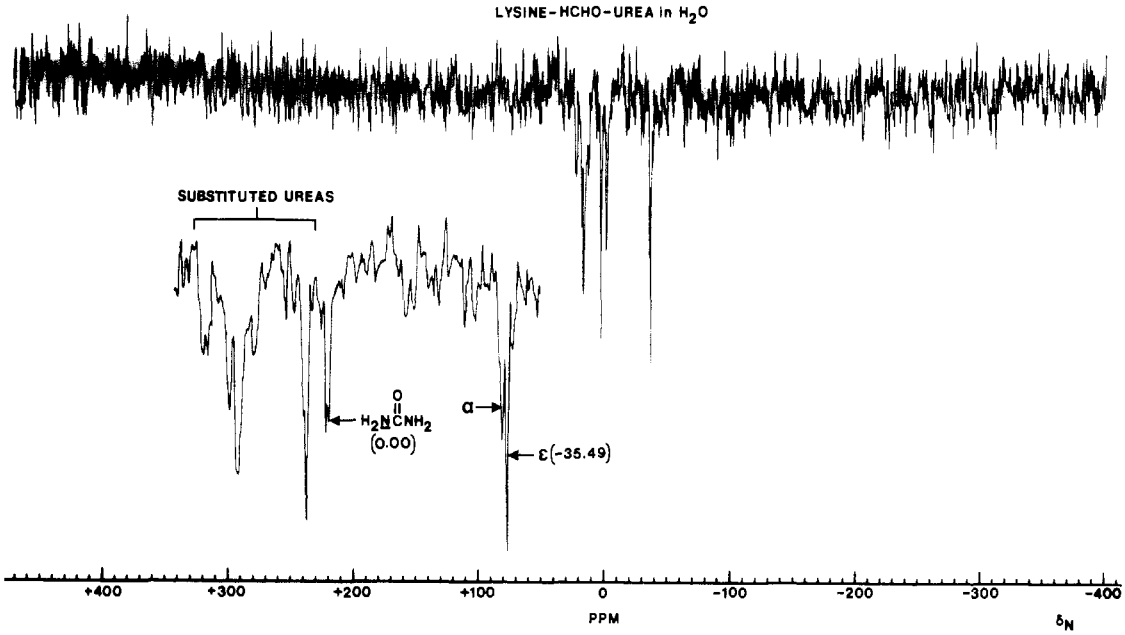

Figure 2. The 7200-Hz ^{15}N spectrum of the lysine–formaldehyde–urea polymer. The ϵ-nitrogen shows a downfield shift to -35.49 ppm while the α-nitrogen of lysine remains essentially unchanged at -34.41 ppm. The other peaks from 0.4 to 20.0 ppm are for mono- and dimethylene-substituted ureas.

vorably with 332.0 and 347.7 ppm, respectively, which were calculated by Witanowski et al. (1973) for lysine dihydrochloride from data reported by Pregosin et al. (1971). These authors originally referenced their chemical shifts to 5 M NH_4NO_3 and reported values of 21.5 and 5.8 ppm for the α- and ϵ-nitrogens. These shifts were later verified by Hawkes et al. (1975), who reported 21.9 and 5.8 ppm for the α- and ϵ-nitrogen of lysine dihydrochloride. Deviations of our ϵ-nitrogen chemical shift can be attributed to the use of the monohydrochloride rather than the dihydrochloride of lysine and a pH of 6.4.

Figure 1 is the spectrum of lysine hydrochloride in H_2O showing two lines separated by 68.36 Hz (7.528 ppm). Figure 2 is a spectrum of the lysine–formaldehyde–urea polymer (pD 6.8). The resonance for the ϵ-nitrogen of lysine has been moved downfield 6.67 ppm while that for the α-nitrogen remains essentially unchanged. The ^{15}N spectra do not have the same signal/noise (S/N) ratio as the ^{13}C spectra reported earlier (Barton et al., 1979). This reduced S/N is due to the lower abundance of ^{15}N than ^{13}C (0.365% vs. 1.108%) and a lower sensitivity (1.04×10^{-3} vs. 1.59×10^{-2}) with respect to the proton. Even with Fourier transform, the level of the signal for ^{15}N is roughly 50 times weaker than that of the ^{13}C signal, necessitating long-term accumulation (10–24 h). Thus, the expected 10–15% unincorporated ϵ-nitrogen, which would be observed at -42.16 ppm, would not afford a signal readily discernible from background noise.

Since ^{15}N has a negative magnetogyric ratio of -9.867 with respect to ^1H, the maximum nuclear Overhauser effect

(NOE) is −3.93 (Witanowski and Webb, 1973). The signal to noise (S/N) enhancement will be maximum if dipole–dipole interactions dominate the relaxation mechanism. If the dipole–dipole interactions are not dominant, the NOE enhancement factor will range from 1 to −3.93, proportional to the magnitude of the dipole–dipole interactions' contribution to the relaxation mechanism. In general, a single bond dipole–dipole interaction gives rise to maximum NOE enhancement. In Figure 2, the α- and ϵ-nitrogen would be expected to have identical intensities as they do in Figure 1. The α-nitrogen signal, however, is only half that of the ϵ-nitrogen. The binding of lysine in the polymer may alter the importance of the various relaxation mechanisms. The ϵ-nitrogen bound in the matrix has intramolecular dipole–dipole interactions, whereas the α-nitrogen has only the intermolecular interactions which are limited in the polymer. These NOE differences are useful for deducing structural changes but make quantitation difficult. Numerous other peaks occur downfield from the urea reference and probably are from urea substituted with methylene derived from formaldehyde. No assignments have been made yet, but one could speculate that the peaks occurring at 0.4 and 1.0 ppm are due to unsubstituted urea nitrogen resonances. The peaks between 4.9–5.6 and 18.7–20.6 ppm likewise could be assigned to mono- and disubstituted urea nitrogen resonances, respectively, since substitution of electron donors larger than methyl is known to cause downfield displacement of the nitrogen resonance in ureas and amines (Witanowski et al., 1973).

ACKNOWLEDGMENT

We gratefully acknowledge M. J. Albright and R. O. Obenauf of JEOL, Cranford, NJ, for taking the ^{15}N spectra on their instrument.

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Received for review April 21, 1980. Revised August 12, 1980. Accepted January 15, 1981. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be sufficient.

Chemical Preservation of Protein in Industrial Whole Animal Blood

Fresh industrial whole animal blood samples obtained from a packer were found to be low in nonprotein nitrogen (~500 mg of N/L) but were contaminated by microbes. Industrial blood samples were incubated for 48 h at 35 °C in the presence of putative chemical preservatives, and the nonprotein nitrogen levels of the samples were compared to those of controls. Chemicals tested were sodium bisulfite, sodium polyphosphate, succinic acid, propyl gallate, benzoic acid, D-isoascorbic acid, propionic acid, sorbic acid, sulfuric acid, acetic acid, and phosphoric acid. Sodium bisulfite or sodium polyphosphate added as solids without pH adjustment to a final concentration of 0.8–1.0 g/100 mL was most effective in preventing blood protein degradation to nonprotein nitrogen. Phosphoric acid added to a final concentration of 0.7 g/100 mL, acetic acid (0.5 g/100 mL), propionic acid (0.75 g/100 mL), and succinic acid (0.75 g/100 mL) were also effective blood protein preservatives.

Fresh whole animal blood, derived from bovine and porcine species, is characterized by low nonprotein nitrogen levels relative to the total nitrogen content. Blood samples obtained from renderers are often high in nonprotein nitrogen and ammonia levels, reflecting considerable degradation of blood protein (Vandegrift and Ratermann,

0021-8561/81/1429-0671$01.25/0