to soil would have to be dictated by its rate of microbiological decomposition in order to avoid establishing an unfavorable carbon to nitrogen ratio for proper fertility and plant growth. The rate of application (10% by weight) used here probably represents an exaggerated level considering what might normally be incorporated in soil if paper is used as a mulching material. Realistically, however, one must take into account the possible accumulation of lead if smaller amounts of such paper were to be repeatedly incorporated in the same soil in succeeding years.

ACKNOWLEDGMENT

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^{13}C Nuclear Magnetic Resonance of the Amorphous Polymer of Lysine–Formaldehyde–Urea

Franklin E. Barton, II,* David S. Himmelsbach, and Henry E. Amos

The structure of an amorphous polymer of lysine–formaldehyde–urea was studied both as a solid and in solution with ^{13}C NMR. A peak at 44.6 ppm was assigned to the ϵ-carbon of lysine whose nitrogen was bound to a methylene or other substituent derived from formaldehyde. The ratios of bound and free lysine were determined for two different polymer preparations. The percentage free lysine in 2.5-day-old material was 65% for the polymer made with lysine monohydrochloride and 21% regardless of age when an equivalent amount of sodium hydroxide to neutralize the hydrochloride was added to the reaction mixture. Assignments of the chemical shifts were made by comparison with known literature values, model systems, and calculations of chemical shifts based on substituent parameters. Hydrolysis of the polymer in 1.0 N DCl under conditions similar to those of a ruminant abomasum yielded free lysine. ^{13}C NMR was shown to be a useful tool for the structural elucidation of the solid and that quantitative information can be obtained from the ^{13}C NMR spectrum of a solid.

Ruminant animals are unique among mammals because of the bacteria and protozoa in the ruminoreticulum which can utilize the energy from digested fibrous plant materials for synthesis of microbial protein. As such, ruminants can utilize energy from feedstuffs that man cannot digest. However, the quantity of microbial protein synthesized within the rumen may not be sufficient to meet ruminant requirements for growth and milk production (Hungate, 1965). Furthermore, microbial protein and many of the common protein supplements are deficient in one or more of three amino acids, i.e., methionine, lysine, and threonine. Nimrick et al. (1970a,b) found that for lambs microbial protein was deficient in these three amino acids. Also lysine and methionine were first and second limiting, respectively, or colimiting amino acids for dairy cows fed conventional diets (Schwab et al., 1976). Performance of growing cattle has been improved by abomasal infusions of a mixture of essential amino acids (Chalupa et al., 1973), lysine (Boila and Deulin, 1972; Deulin and Woods, 1964) and methionine (Steinacker et al., 1970; Fenderson and Bergen, 1975).

Supplementation of ruminant diets with free amino acids has not been practical because they are rapidly degraded by the rumen microorganisms (Broderick, 1975; Chalupa, 1976). Accordingly, several attempts have been made to protect amino acids from rumen degradation and have met with various degrees of success (Amos et al., 1974; Richardson et al., 1976; Langer et al., 1975).

This report describes the structure of a white amorphous polymer of lysine–formaldehyde–urea as determined by ^{13}C NMR. This polymer has been shown to protect about 55% of the lysine from rumen microbial attack, without affecting its biological availability (Amos and Evans, 1978a,b).

EXPERIMENTAL SECTION

Reagents. L-Lysine monohydrochloride, urea, and formalin (37% formaldehyde) were obtained as reagent

Field Crops Utilization and Marketing Research Laboratory, Richard B. Russell Agricultural Research Center, U.S. Department of Agriculture, Science and Education Administration, Athens, Georgia 30604.

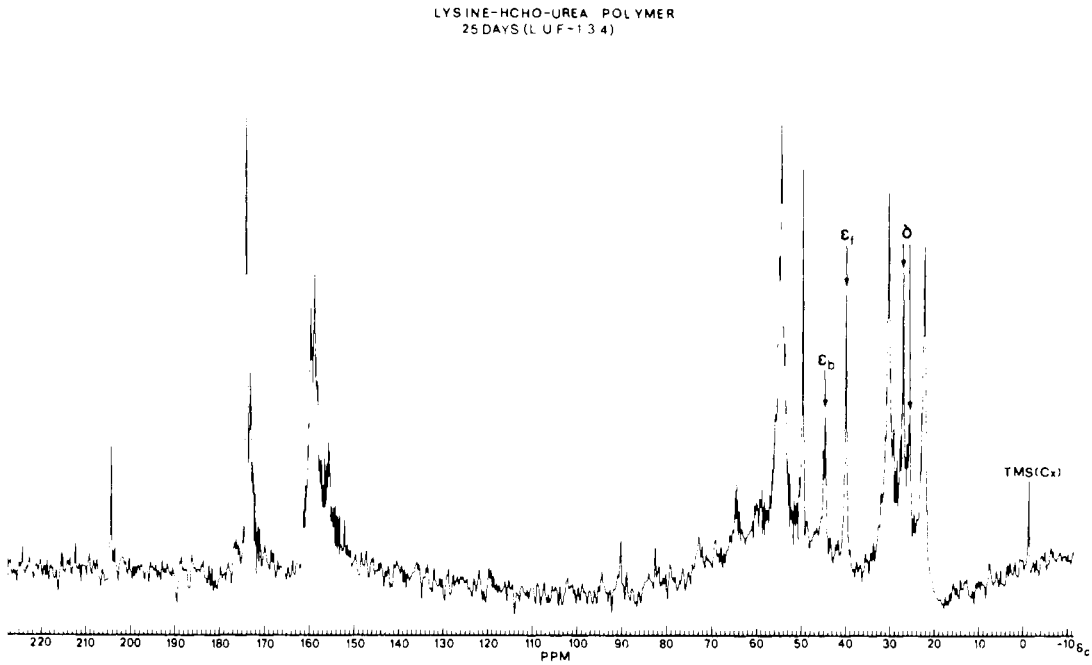

Figure 1. The 6000-Hz ^{13}C spectrum of the solid lysine–formaldehyde–urea (LFU) polymer. The polymer was formed using a lysine/urea/formaldehyde ratio of 1:3:4. The spectra was taken 2.5 days after preparation.

grade chemicals and were not purified further. Enriched (^{13}C, 90 atom %) formalin was obtained from Merck Sharp and Dohme, Canada, and mixed with the reagent grade formalin in the proper molar ratio to have a 30-fold ^{13}C enrichment.

Preparation of Lysine–Formaldehyde–Urea Polymer. The lysine–formaldehyde–urea polymer was prepared in one of several ways. (1) Lysine monohydrochloride was added to a urea–formalin solution in 1:3:4, 1:2:3, or 1:1:2 molar ratio of lysine/urea/formaldehyde (l:u:f). (2) Alternatively the three reagents were mixed simultaneously in the above ratios. (3) One equivalent of sodium hydroxide was added with the lysine monohydrochloride. In these experiments the molar ratio of lysine/urea/formaldehyde was 1:3:4.

In method 1 and 2 the polymer was cut into plugs after hardening and placed into a 10-mm NMR tube. A 5-mm coaxial cell containing the lock material (deuterioacetone or deuterium oxide) was then placed in a 5-mm hole bored in the center of the plug, and the spectra were recorded in 1–2 h (512–8192 scans). Alternatively, the reactions were run by methods 1, 2, or 3 in a 10-mm NMR tube and the formation of the polymer and changes in its structure were monitored with time. Times of 2–5 h, 2 days, 4 days, and 4 weeks were used routinely, this last value being a time infinity measurement. In these experiments the tube and coaxial cell were not salvageable as they fused with the polymer. The neutralized samples were freeze-dried to remove excess formaldehyde. This gave a white, very hygroscopic solid which could be dissolved in D_2O for ^{13}C NMR evaluation.

NMR Parameters. Carbon-13 spectra were obtained on a JEOL ^{1}PS/PFT 100, pulsed NMR spectrometer. A Nicolet 1083 (20 bit word) computer system was interfaced to the spectrometer to collect the free induction decay (FID) and perform the fast Fourier transform (FFT) and integration of peak areas. The observing, lock, and irradiating frequencies were calibrated with a Dana 8010B frequency counter. Line positions were referenced to tetramethylsilane (Me_4Si) in a coaxial cell at −42.7 Hz or sodium trimethylsilyl propionate (TSP) at −34.2 Hz with respect to Me_4Si at 0 Hz. Hertz measurements are calculated by the computer and are accurate to the line width described by one data point (0.8 Hz). The instrument was tuned before each spectrum and locked on the deuterium of deuterioacetone or deuterium oxide. Each spectrum was taken with a 6000-Hz bandwidth, and 8K data points were assigned to the FID unless otherwise noted. All spectra were taken with a 90° pulse angle of 25 μs pulse width. Pulse repetition rate was 3.0 s (unless otherwise stated), and temperature was 25 °C.

Hydrolysis. Samples of the polymer were hydrolyzed in 1.0 N DCl at 39 °C in the NMR tube to approximate abomasum conditions, and the NMR spectra were recorded as described above.

RESULTS AND DISCUSSION

The chemical shifts of the methylene and methine carbons of lysine as they occur in the various environments created by the reaction system are given in Table I. The lysine–formaldehyde–urea (pD 1.0) column shows the effect of pH on chemical shift. These observed shifts agreed with those reported by Saito and Smith (1973). It was interesting to note that the chemical shift of the ϵ-carbons in free lysine entrapped in the solid polymer had the same chemical shift (39.9 ppm) as the ϵ-carbon in solution spectra of lysine hydrochloride. The lysine–formaldehyde adduct was a milky, viscous solution and formed only on the ϵ-carbon (i.e., no α-carbon shift).

The spectrum of the typical lysine–formaldehyde–urea polymer (i.e., 2.5 days old, l:u:f is 1:3:4) is shown in Figure 1. The principal significance of this spectrum (Figure 1) is that it was taken with the polymer as a solid. Since neither "magic angle" spinning nor cross-polarization techniques were used, it is assumed the polymer was amorphous. The molecular weight of the polymer material was not determined. The resonances between 20 and 55 parts per million (ppm) were essentially the methylene and methine carbons in lysine. Particular attention is directed to the ϵ- and δ-carbon resonances. Both carbons are sensitive to substitution on the ϵ-nitrogen of lysine. As would be expected the change in chemical shift for the ϵ carbon (4.8 to 4.7 ppm) is more pronounced and in opposite direction from the δ-carbon shift (−1.4 to −0.7 ppm).

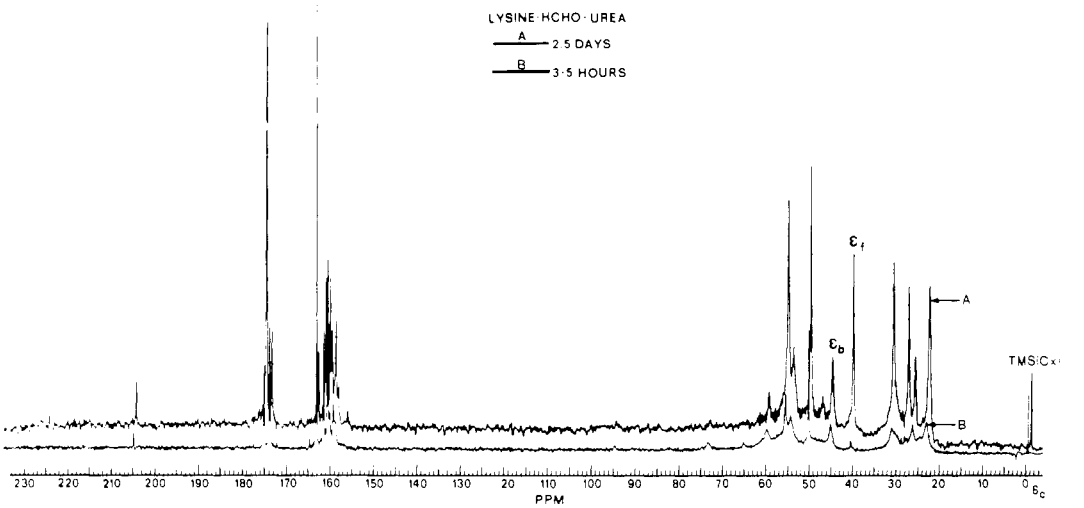

Figure 2. The 6000-Hz ^{13}C spectra of the solid LFU polymer at 2.5 days (A) and initially at 3.5 h (B).

Table I. Chemical Shift (δ in ppm from Me_4Si) for Methylene and Methine Carbons of Lysine in the Lysine-Formaldehyde-Urea System (at the pD of the Monohydrochloride in D_2O)

carbon	LFU[a] (PD 1.0)	LFU (Lys·HCl)	LF[b]	Lys[c]	N^{ϵ}-ME-Lys[d]
Lys α	55.1	54.8	55.0	55.2	55.2
Lys β	29.8	29.9	29.9	30.6	30.7
Lys γ	22.1	21.5	22.2	22.3	22.4
Lys δ	25.4 (26.8)[e]	25.6 (26.3)[e]	25.5	27.2	25.9
Lys ϵ	44.4 (39.6)[e]	44.6 (39.9)[e]	43.2	39.9	49.3

[a] LFU is lysine-formaldehyde-urea polymer. [b] LF is lysine-formaldehyde-lysine. [c] Lys is lysine monohydrochloride. [d] N^{ϵ}-Me-Lys is N^{ϵ}-methyllysine. [e] Numbers in parentheses are the chemical shifts for the free lysine present that is entrapped in the polymeric material.

Table II. Peak Area Integrations and Intensities for Bound and Free Lysine in Lysine-Formaldehyde-Urea Polymer

	using lysine hydrochloride (1:3:4) (2.5 days after prep.)				
	int	%	area	%	av %[a]
ϵ_f[b]	127.7	70.6	93.0	57.4	64.0
ϵ_b	52.9	29.4	68.9	42.6	36.0
δ_f	105.0	66.6	96.4	66.6	66.6
δ_b	52.6	33.4	48.4	33.4	33.4

[a] Average percent substitution of the lysine = 34.7%; free lysine = 65.3%. [b] Subscript f indicates the carbon is in free lysine while b indicates the carbon is in bound lysine.

These resonances can be used to approximate the amount of lysine that is bound in the polymer matrix. Peak intensities and area integrations with complete decoupling are not precisely quantitative due to differences in field strength, spin–lattice relaxation times (T_1's), and the nuclear Overhauser effect (NOE). However, the intensities do give approximately correct relative values when the same carbon is considered and the three factors (field strength, T_1, and NOE) are about equal (Barton et al., 1975). The spectral region from 55 to 90 ppm contained resonances attributed to methylenes derived from formaldehyde. The resonances from 157 to 206 ppm were attributed to carbonyls from urea, formaldehyde, lysine, and acetone (from acetone-d_6 in coaxial cell), respectively. The ^{13}C NMR spectrum of the polymer when hydrolyzed with 1.0 N DCl at 39 °C was that of free lysine. The hydrolysis experiment was necessary to determine whether lysine or a derivatized lysine was the product. Neuberger and Sanger (1944) found that N^{ϵ}-methyllysine had the same feeding value for rats as lysine. To date little evidence exists for ruminants. Amos and Evans (1978a,b) found that in rats the polymer has the same feeding value as a diet containing the same level of lysine and that in sheep (Amos et al., 1978) the polymer has the same feeding value as the comparable level of lysine infused into the abomasum after corrections for the amount degraded intrarumenally.

Peak intensities and area integrations indicated that in the solid polymer (2.5 days old, l:u:f 1:3:4) about 35% (Table II) of the lysine is bound with a methylene on the nitrogen on the ϵ-carbon. The resonance at 44.6 is the ϵ-carbon shifted downfield because of the methylene substitution on the nitrogen. The amount of bound lysine decreased with time. When the polymer was approximately 3 months old it contained essentially free lysine entrapped in a urea–formaldehyde resin. This lack of long-term stability could be associated with acid-catalyzed reversal of the Mannich type reaction which formed the polymer (House, 1965). The acid in this case was derived from the hydrochloride of lysine hydrochloride. Although the lysine was essentially free, it was still entrapped in the polymer matrix. It is reasonable to expect that some of the entrapped lysine would not be solubilized in the rumen and degraded by the microorganisms and would pass into the abomasum where it could be utilized. Several changes in experimental procedure was made, however, in an attempt to increase stability. The molar ratio of lysine to urea to formaldehyde was made 1:1:2 and 1:2:3 by method 1. Figure 2 shows the spectra of the polymer initially (B) and after 2.5 days of (A) for the 1:1:2 ratio. Initially the amount of bound lysine was greater than the amount of free lysine as evidenced by a larger resonance at 44.6 ppm than at 39.9 ppm (see Figure 2); but after 2.5 days free lysine predominated. In general, urea was added to the formalin, and lysine hydrochloride was then added to this cold solution. Several experiments were run in which all reagents were mixed simultaneously at 1:1:2 and 1:3:4 molar ratios of lysine–urea–formaldehyde. The results were essentially the same for all ratios; the initial binding of lysine was high but decreased with time.

An alternative approach was to neutralize the lysine hydrochloride as it was mixed with the urea formalin solution. This method produced a material with different physical properties. Although it too was a white

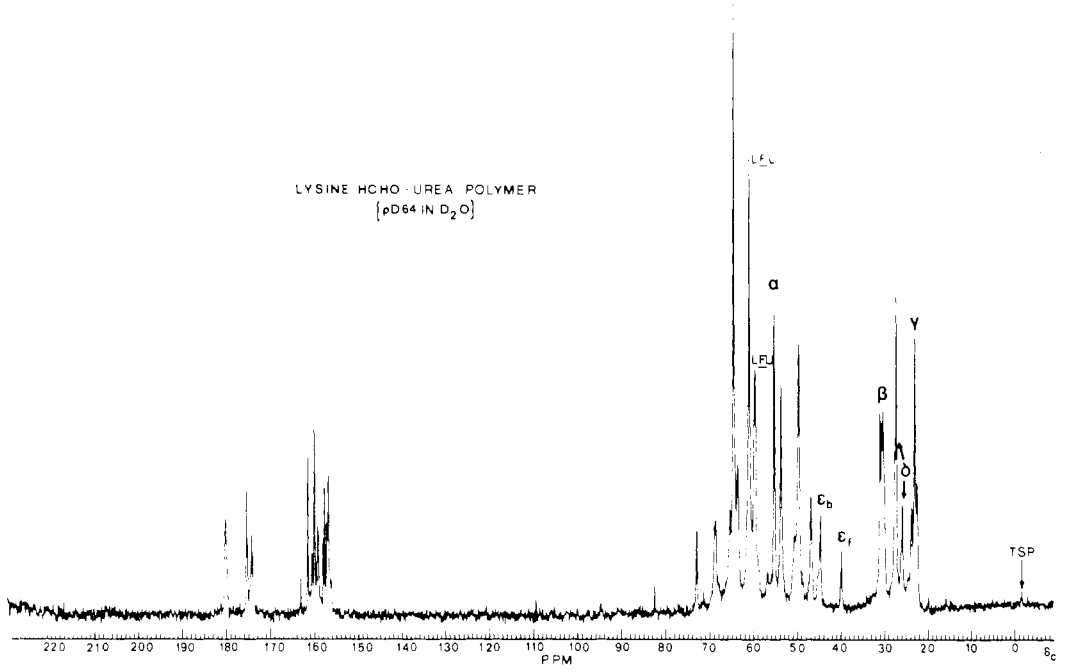

Figure 3. The 6000-Hz ^{13}C spectrum of the LFU neutralized polymer in D_2O at pD 6.4.

amorphous solid, it was very hygroscopic and completely soluble in water (or D_2O). The spectrum of this material was therefore much better resolved (Figure 3). Resonances at 56.6, 59.4, and 60.8 ppm were assigned to the methylenes between two ureas (UFU), one lysine and one urea (LFU), and two lysines (LFL), respectively, based on substituent effect calculations and the work of Saito and Smith (1973), Kelly et al. (1977), and Dewar et al. (1975). There was additional β-, γ-, and δ-lysine methylene signals (e.g., those at 27.6 and 22.5 ppm and the two β signals, Figure 3). The extra methylenes were attributed to the β-, γ-, and δ-lysine carbons of poly-L-lysine. The carbonyl signals at 174, 175.5, and 180 ppm were assigned as nonterminal lysine and free formaldehyde (174 ppm), N-terminal lysine and free lysine (175.5 ppm), and C-terminal lysine (180 ppm), respectively, for poly-L-lysine and free lysine (Saito and Smith, 1973). Resonances at 65.0, 71.5, and 73.0 ppm were assigned to mono-, di-, and tetramethylol substitution on lysine or urea based on the work of Slonim et al. (1977). These workers prepared model compounds of urea–formaldehyde adducts and made the methylol assignments in the 63–73-ppm region of the spectrum. The addition of lysine to the system complicates the problem of assignments. As such our assignments are tenuous.

When the polymer was made with formaldehyde enriched with 33% (v/v) of 90 atom % [^{13}C]formaldehyde the spectrum shown in Figure 4A was obtained. Comparison of both spectra enabled us to ascertain those resonances attributable to a methylene or methylol derived from formaldehyde, lysine, or urea.

Assignments were made by comparison with literature values, calculations from model systems and substituent constants, comparison with known compounds, and the comparison of the individual components of the lysine–formaldehyde–urea polymer with the results obtained using the reagents in pairs. This approach allowed us to make composite spectra so that regions of similarity could be directly compared. The chemical shifts obtained in this manner are shown in Table I. It was by this method and that of ^{13}C enrichment that the resonance at 44.6 ppm was assigned to the ϵ-carbon of lysine in the polymer. The lysine resonances were assigned by comparison with the results of Saito and Smith (1973), Christl and Roberts (1972), and Johnson and Jankowski (1972). The carbonyl resonances were assigned according to Saito and Smith (1973) for both L-lysine and poly-L-lysine. It is doubtful that the poly-L-lysine was larger than the trimer since the intensities of all three carbonyl signals were similar.

Three resonances required some special consideration before assignments could be made. These were for the methylenes between two lysines, two ureas, and one lysine and one urea. The work of de Breet et al. (1977) has shown that methylenediurea (MDU) can be used as a model system and that from its methylene chemical shift other methylene chemical shifts can be calculated with the use of the additivity increments for substituent effects (Stothers, 1972). Using 47.1 ppm, the reported chemical shift for methylenediurea, a chemical shift of 55.7 ppm was calculated for a methylene (*F*) between two ureas in a resin (i.e., U-F-U-*F*-U-F-U). The resonance at 56.6 ppm was therefore assigned to a methylene between two ureas in a polymer matrix (Figure 4, UFU). Dewar et al. (1975) reported the chemical shift of the central methylene between the two nitrogens in hexahydropyrimidine to be 61.1 ppm. Since the methylene between two lysines would be an analogous methylene, the peak at 60.8 ppm was assigned to a methylene between two lysines (Figure 4, LFL). If the additivity increment for oxygen (2 × 6.2) substitution on the carbons γ to the L*F*L methylene is used to change this to methylenediurea, a predicted value of 48.7 ppm is obtained for methylenediurea starting from the hexahydropyrimidene or 48.4 ppm if the observed shift of 60.8 ppm is used. This value is close to the 47.1 ppm value of Saito and Smith (1973) for MDU and given good approximation for L*F*L by two means. Recent work of Kelly et al. (1977) on the cross-linking of amino acids with formaldehyde estimate the methylene in L*F*L to be 64 ppm. Thus the downfield 60.8 ppm should be the methylene in L*F*L. A closer approximation of the LFL methylene may be obtained by considering *N*-methyllysine as the model compound rather than the hexylamine model used by Eggert and Djerassi (1973). It was from this study that Kelly et al. (1977) estimated the value of 64 ppm for LFL. Using the *N*-methyllysine model and correcting for

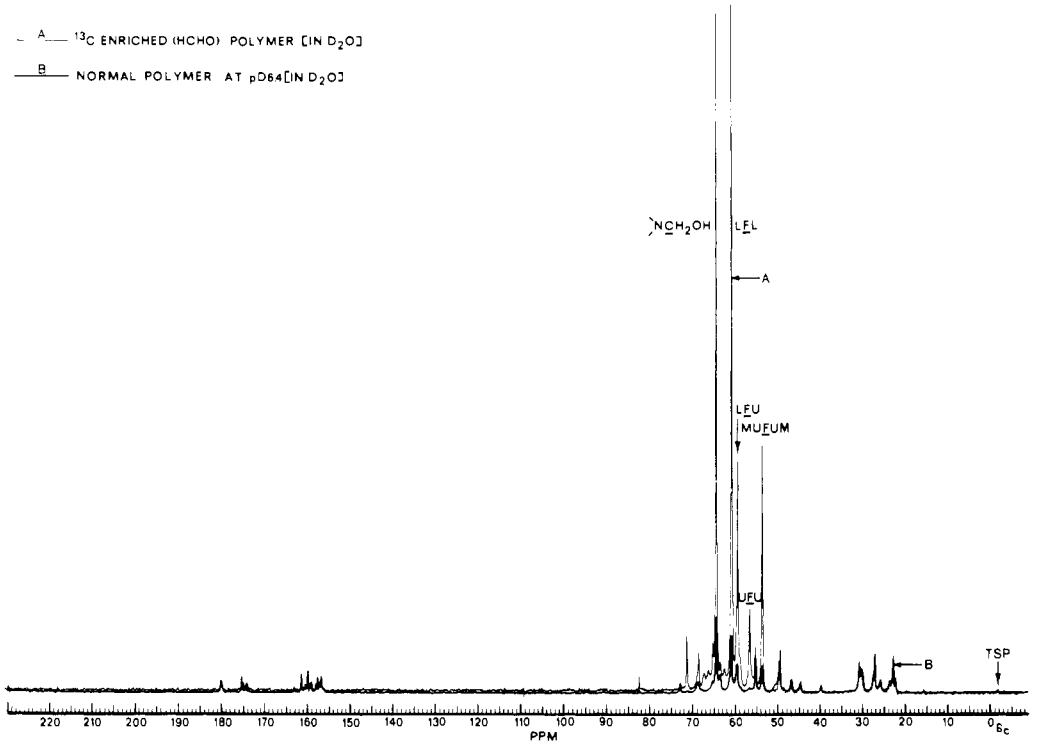

Figure 4. The 6000-Hz ^{13}C spectrum of the 90% ^{13}C enriched (33-fold ^{13}C enrichment) LFU neutralized polymer (A) superimposed on the spectrum of the neutralized polymer (B). Both spectra are in D_2O at pD 6.4. The resonance at 53.6 ppm could be a methylol substituted on UFU.

Table III. Peak Area Integrations and Intensities for Free and Bound Lysine in the Neutralized Lysine-Formaldehyde-Urea Polymer

	lysine hydrochloride (1:3:4) with 1 equiv of NaOH added[a]				
	int	%	area	%	av %[b]
ϵ_f[c]	22.7	24.4	73.2	13.4	18.9
ϵ_b	70.2	75.6	469.5	86.5	81.1
δ_f	28.5	25.6	100.5	20.6	23.1
δ_b	82.9	74.4	387.6	79.4	76.9

[a] From spectrum after 3 weeks and at 1 h after beginning of hydrolysis at pD 1.0. [b] Average substituted lysine = 79.0%; free lysine = 21.0%. [c] Subscript f is for the carbon in free lysine while b denotes the carbon in bound lysine.

a solvent shift (subtract the average solvent shift for lysine in LFU and *N*-methyllysine in D_2O of the δ-, β-, and γ-methylenes in Table I) of 0.9 ppm, the calculated value of 61.5 ppm is obtained. If the LFU methylene is assumed to be intermediate between L*F*L and U*F*U and an average of the hexahydropyrimidene and the calculated UFU chemical shifts [(61.1 ppm + 55.7 ppm)/2] is taken, 59.85 ppm is the calculated value. Therefore the peak at 59.4 ppm was assigned to a methylene between a lysine and a urea in the polymer matrix (Figure 4, LFU). These three peaks also showed the dramatic increase expected in the enrichment experiment (Figure 4A).

Table III gives peak intensities, area integrations, and calculated percentages of free and substituted lysine for the neutralized material. In this case the lysine was 79% bound and 21% free. This percentage did not change over a period of 5 months. Upon hydrolysis at pD 1.0 at 39 °C, the polymer disappeared and only free lysine remained, indicating the polymer would be available to the animal in the abomasum. The results of in vitro rumen fermentation (Amos and Evans, 1978a,b) with lysine hydrochloride polymer were that 58% of the lysine escaped degradation in the rumen. This figure (58%) is larger than the 35% average for the bound lysine in Table II. Thus some free lysine entrapped in the matrix must escape degradation by rumen microorganisms. The neutralized polymer shows evidence of being even more stable to the conditions of the rumen.

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Direct Determination of Phosphorus in Fertilizers by Atomic Absorption Spectroscopy

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Phosphorus (expressed as P_2O_5) content in commercial fertilizers has been determined by the method of atomic absorption spectroscopy (AAS). The application of the technique takes advantage of the low background absorption by the premixed nitrous oxide–acetylene flame and the phosphorus nonresonance line at 213.6 nm. Phosphorus in eight commercial fertilizers, with reported percentages ranging from 10 to 46 and two National Bureau of Standards phosphorus standards, was determined with excellent precision and accuracy. No significant spectral, matrix, or chemical interferences were observed. The AAS method of determining phosphorus in fertilizer has the advantage of speed, specificity, and freedom from interferences.

Currently, phosphorus in fertilizers is routinely determined either by the gravimetric quinolinium molybdophosphate or the molybdovanadophosphate spectrophotometric methods (AOAC, 1975). Both of these methods share certain drawbacks. The spectrophotometric method is not applicable to fertilizers that are either colored or that yield colored solutions or contain ions other than orthophosphate which form colored complexes with molybdovanadate reagent. This is a major limitation since a substantial number of fertilizers are colored. The gravimetric method, in spite of its accuracy and precision, is inherently slow; it involves several steps and also requires several reagent solutions. Furthermore, both methods require that phosphorus be in the form of orthophosphate. This requirement calls for elaborate digestion steps except for the determination of water-soluble phosphates. In view of the limitations inherent in the techniques currently available for phosphorus determination in fertilizer, we have investigated and established the use of an atomic absorption method. The method is rapid (requiring minimal chemical manipulation and no separation), specific for phosphorus, and free from interferences.

The determination of phosphorus by atomic absorption spectroscopy (AAS) has been difficult to achieve in the past because the ground-state lines of phosphorus lie at wavelengths less than 200 nm, the region where flames and atmospheric gases such as oxygen absorb light very strongly. Therefore, in order to measure phosphorus directly at its ground-state lines, the flame employed must not only be hot enough to atomize the phosphorus efficiently, but must also be transparent to the primary source at these wavelengths. Nitrogen purging of the optical path is also necessary to remove oxygen. In practice this is difficult to accomplish.

These problems prompted Manning and Slavin (1969) to investigate the use of the premixed nitrous oxide–acetylene flame in the determination of phosphorus at its nonresonance line of 213.6 nm. The flame showed marked reduction in noise and background absorption. In addition, the need for nitrogen purging of the optical path was eliminated by performing measurements at this wavelength. The major disadvantage in using this line was due to poor sensitivity (290 μg/mL). In spite of this limitation, however, the method was highly reproducible for the direct determination of phosphorus in high concentrations. Furthermore, Kerber et al. (1970) established that AAS determination of phosphorus was practically free from chemical interferences. On the basis of all this information we decided to explore the potential applicability of this technique to analytical determination of samples containing large amounts of phosphorus, such as fertilizers. Our findings are the subject of this report.

EXPERIMENTAL SECTION

Apparatus. A Perkin-Elmer Model 103 atomic absorption spectrometer was used in conjunction with a phosphorus hollow cathode lamp and a nitrous oxide burner head (Perkin-Elmer No. 303-0419). The fuel and the oxidant flow rates were set respectively at 9 and 16 L/min. Once the flame was ignited, the fuel flow was adjusted until the rose-red inner cone was about 2 cm high. Expansion was kept at zero for all the determinations to limit noise from the detector. It was found necessary to clean the burner from time to time in order to remove solids that accumulate on it due to large amounts of dissolved solids.

Reagents. All chemicals used for preparing standard solutions and for the interference studies were of reagent

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