nearly linear. The different spatial averages are all about the same. Only a few special orientations may not fit this averaging pattern, thereby resulting in a slight curvature. That is, nonlinearity in $T_{1\rho}(C)$ can arise from contributions from those methylene carbons having a carbon–proton internuclear vector lying along the sample rotation axis. Carbons approximately satisfying this condition will have relatively long $T_{1\rho}(C)$'s which will remain longer than the average for the whole sample even with magic-angle spinning.

Regardless of the details of the orientational averaging, the observation remains that relative methylene-carbon $\langle T_{IS}(ADRF)\rangle$'s can be calculated if the corresponding H_L's are known. This means estimates of spin–spin contributions to methylene-carbon $\langle T_{1\rho}(C)\rangle$'s for any polymer can be made without recourse to elaborate nine-step ADRF procedures. A proton T_2 or a JBSLF determination of H_L, in combination with the use of eq 8, may be sufficient. The more elaborate accurate determination of the ratio of $\langle T_{1\rho}(C)\rangle$ to $\langle T_{IS}(ADRF)\rangle$ can then be reserved for particularly difficult cases.

Estimates of $\langle T_{IS}(ADRF)\rangle$ for other types of carbons are also possible. Thus, the 7-kHz H_L for the protonated aromatic carbon of poly(α-methylstyrene)[23] suggests a $\langle T_{IS}(ADRF)\rangle$ of about 70 ms (eq 9). A more accurate estimate would probably result if parameters from an aromatic reference compound were used in eq 9 rather than those from glycine. Nevertheless, it is clear that the observed $\langle T_{1\rho}(C)\rangle$ at 37 kHz of 19 ms (Table V) must be dominated by spin–lattice contributions.

Registry No. Glycine, 56-40-6; polyethylene (homopolymer), 9002-88-4; dipalmitoylphosphatidylcholine, 2644-64-6; poly(α-methylstyrene) (homopolymer), 25014-31-7; polystyrene (homopolymer), 9003-53-6; hexadecane–urea clathrate, 3311-88-4; poly(*o*-bromostyrene) (homopolymer), 27290-16-0; isotactic polystyrene (homopolymer), 25086-18-4.

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Double-Cross-Polarization Detection of Labeled Chemical Bonds in HCN Polymerization

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ABSTRACT: Hydrogen cyanide reacts in the presence of base to form a complex mixture of HCN polymers. The structure of these polymers and the mechanism of their formation, especially the insoluble, higher molecular weight ones, have been disputed. It is of particular interest to know whether significant amounts of heteropolypeptides are present in these materials following hydrolysis in cold water. We have used CPMAS ^{15}N NMR previously to establish the presence of amide nitrogens in the insoluble hydrolysate. Now we report the use of double-cross-polarization (DCP) NMR to show that most of these nitrogens are found in peptide linkages. DCP NMR involves the sequential transfer of spin polarization from ^{1}H to ^{15}N to ^{13}C followed by the detection of ^{15}N under MAS conditions. The ^{15}N to ^{13}C transfer requires strong dipolar coupling between the spins and hence detects ^{15}N–^{13}C chemical *bonds* when compared with CPMAS ^{15}N NMR. Reactions of $H^{13}C^{15}N$ and $H^{12}C^{14}N$ *or* $H^{13}C^{14}N$ and $H^{12}C^{15}N$ to form $(HCN)_x$ have been examined with DCP NMR to detect the making or breaking of ^{15}N–^{13}C bonds to find evidence for peptide linkages and other structures.

Introduction

The combination of high-power resonant decoupling and high-speed mechanical sample spinning at the magic angle has been shown to produce liquidlike, high-resolution ^{13}C and ^{15}N NMR spectra of organic solids.[1-3] The sensitivity of these rare-spin NMR experiments is improved by cross-polarization transfer of spin polarization from the abundant protons to the rare spins.[4] One of the principal

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uses of the combined cross-polarization, magic-angle spinning (CPMAS) technique is in the characterization of intractable materials, substances which are insoluble and infusible and opaque to radiation at the usual spectroscopic frequencies.

The polymerization of HCN gives rise to such materials.[5] Even in storage, HCN will form a dark brown solid, most of which is insoluble in water or other common solvents. Small amounts of base catalyze this reaction. An examination of these materials, following hydrolysis in cold water, with natural abundance CPMAS ^{15}N NMR reveals a wide variety of nitrogen chemical functionality.[6]

However, we should like to probe beyond discovering the primary environments in which the nitrogen atoms find themselves and learn something about the extended structure of this material. To this end, we have combined two techniques: First, we introduce *both* ^{13}C and ^{15}N labels into the $(HCN)_x$ system by a reaction either between $H^{13}C^{14}N$ and $H^{12}C^{15}N$ *or* between $H^{13}C^{15}N$ and $H^{12}C^{14}N$. Second, we use an additional cross-polarization step in the NMR experiment to detect the existence of ^{15}N–^{13}C *bonds*. That is, for each kind of nitrogen we can identify in the ^{15}N NMR spectrum, we also detect the fraction bonded to ^{13}C atoms. The reaction conditions are mild enough that no N–C bonds are broken until hydrolysis; we know the concentration of ^{15}N–^{13}C bonds in the starting materials. Therefore, any indication of new ^{15}N–^{13}C bonds may tell us more about the structure of the nitrogen we are observing. We call the procedure of sequentially transferring spin polarization from ^1H to ^{15}N to ^{13}C followed by detection of ^{15}N under MAS conditions double-cross-polarization (DCP) NMR.[7-9] The ^{15}N to ^{13}C transfer requires strong dipolar coupling between the spins and hence detects ^{15}N–^{13}C chemical bonds when compared with CPMAS ^{15}N NMR. The use of multiple labels to produce ^{15}N–^{13}C bonds we call chemical-bond labeling.[10-12]

Of the several ways two successive cross-polarization transfers between three-spin systems might be arranged, we have chosen the one easiest to quantify. First, we choose to observe ^{15}N because, as we shall see below, we are interested in a particular nitrogen functionality. Second, we observe the DCP spectrum as a difference between an ordinary ^{15}N CPMAS spectrum (^1H to ^{15}N polarization transfer) and a ^{15}N CPMAS spectrum reduced by contact with the ^{13}C spin system (^1H to ^{15}N to ^{13}C polarization transfer). The second contact drains away ^{15}N polarization at a rate which is dependent on the specific ^{15}N–^{13}C bond(s) and which may be determined from model compounds. During the second contact, the ^{13}C radio-frequency field is phase modulated to prevent buildup of ^{13}C polarization. This maximizes the DCP difference by preventing transfer of ^{13}C polarization back to the ^{15}N spin system and also relieves us of knowing $T_{1\rho}(C)$, the ^{13}C rotating-frame relaxation time. The effect of $T_{1\rho}(N)$ is cancelled if we obtain the DCP *difference* relative to the ordinary ^{15}N CPMAS signal so we need not know $T_{1\rho}(N)$ either. A direct observation of the ^{15}N DCP signal, from a ^1H to ^{13}C to ^{15}N polarization transfer, would require knowledge of $T_{1\rho}(C)$ and $T_{1\rho}(N)$ as well as the drain rate to quantify. Furthermore, an excessively short $T_{1\rho}(C)$ would deteriorate rather than enhance the DCP signal. The DCP difference method is shown in Figure 1. We state the ^{15}N–^{13}C *bonds* are detected in the DCP experiment because the drain rate varies inversely as the 6th power of the internuclear distance so that nuclei in other molecules or two bonds away can ordinarily be neglected.

There is a reason for looking for the presence of a particular extended structure in HCN polymer. It has been

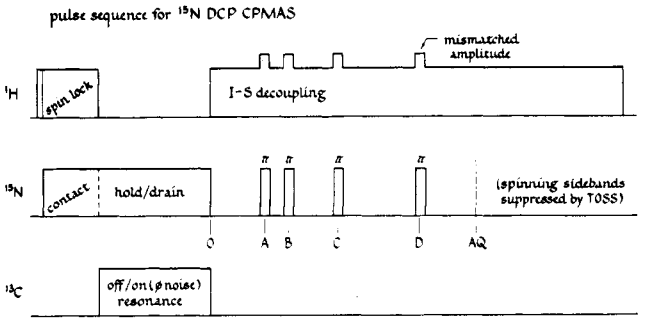
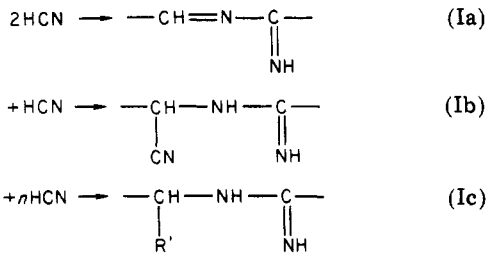


Figure 1. Pulse sequence for double-cross-polarization ^{15}N NMR. The experiment starts with a standard matched spin-lock cross-polarization transfer from protons to nitrogen. The nitrogen magnetization is then either held spin locked or else partially drained by a second matched spin-lock transfer to carbons kept hot by radio-frequency phase modulation. The difference between the hold and drain parts of the experiment gives rise to an NMR spectrum only from those ^{15}N's directly bonded to ^{13}C's. The ^{15}N spectrum can be rendered spinning-sideband free by the use of four refocusing 180° pulses occurring during two magic-angle rotor periods.

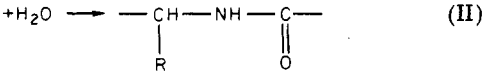
proposed that the original heteropolypeptides on primitive Earth may have formed spontaneously from HCN and water[13-16] without the prior formation of α-amino acids.[17-20] This proposal has been challenged on the grounds that the existence of peptide bonds in hydrolyzed HCN polymer has not been proven. To date, most of the attention has been given to the usually minor fraction (by weight) of the HCN reaction products that are water soluble, without reaching agreement.[21,22] In our earlier study of the insoluble hydrolysate of HCN polymer by natural abundance CPMAS ^{15}N NMR we detected amide nitrogens but were unable to determine whether they were present as peptides or merely side-chain (primary) amides. It is the purpose of this paper to show how the use of chemical-bond labeling followed by double-cross-polarization ^{15}N NMR has revealed the presence of peptide bonds in the insoluble hydrolysate of HCN polymer.

Formulation of Chemical Problem

It has been proposed by Matthews et al.[13-16] that the following series of reactions take place during the base-catalyzed polymerization of HCN:



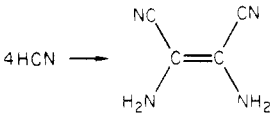
where R′ represents any side chain which may result from the further reactions of HCN and the –CN side chain. Hydrolysis results in the final product



where R represents the result of hydrolyzing R′. At least part of the polymerization may not proceed past the first reaction in this series, which need not be limited to two HCN's (what is pictured may be thought of as a monomer unit). The result of reaction II is a heteropolypeptide formed without the prior formation of α-amino acids. The higher molecular weights are expected to be insoluble in water.

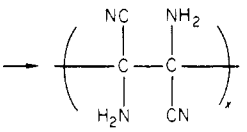
Many other reactions have been proposed in the polymerization of HCN.[5,22] As may be seen from the com-

plexity of the CPMAS ^{15}N NMR spectrum of HCN polymer,[6] many of these reactions probably do take place. One series of reactions capable of producing primary amide nitrogens (as side chains) but no peptide (secondary) nitrogens proceeds through the production of diaminomaleonitrile



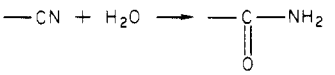
(III)

which may polymerize to produce poly(aminocyanomethylene)



(IV)

Hydrolysis may produce primary amide nitrogens from the side chains

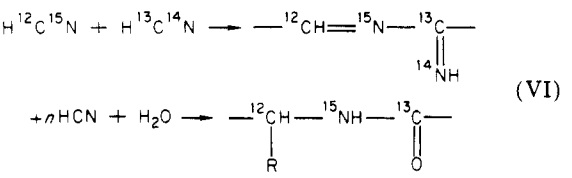


(V)

However, these -CN groups are not particularly reactive and few will hydrolyze in cold water. Indeed, the -CN group of poly(aminomalononitrile)—reaction Ib—is much more active and more likely to react this way, if that group escapes further HCN addition before hydrolysis.

Peptide nitrogens cannot be distinguished from primary amide nitrogens by their chemical shifts since both occur at about 95 ppm (relative to solid ammonium sulfate).[6] However, the peptide nitrogen is unique because it has acquired a new, directly bonded carbon in addition to the one it started with. (We refer to amide nitrogens of this type as "peptide" even though "peptide-like" is a more accurate description.) This places it in the main chain of a (potentially) polymeric material. By contrast, poly(aminocyanomethylene) has no new N–C bonds, no main-chain nitrogens, and cannot hydrolyze to give peptide nitrogens. Thus the search for polypeptides is equivalent to the search for amide nitrogens bonded to two carbons. The fact that we can restrict our attention to the vicinity of 95 ppm permits us to limit our attention to only two structures. (Tertiary amides can usually be expected to occur some 10 ppm farther upfield.[23] Furthermore, an examination of the rate of ^{1}H–^{15}N cross polarization shows, by the absence of slow cross polarizers, that there are no nonprotonated (tertiary) nitrogens reporting at 95 ppm.[6]) It is possible to write down other HCN reactions giving rise to peptide bonds, but the reaction series I and II make the most sense chemically. Of course, any reaction to produce a peptide linkage will have to produce a new C–N bond. In the reaction series given here, the new bond is a N–CO bond. We shall see later than the DCP results make unlikely those reactions giving a new N–CH bond. We therefore treat this possibility only in passing.

We have used two different strategies for introducing ^{15}N and ^{13}C labels into $(HCN)_x$. In both cases DCP methods were then used to detect the presence and concentration of ^{15}N–^{13}C bonds. The simplest strategy involves a reaction between equimolar amounts of $H^{13}C^{14}N$ and $H^{12}C^{15}N$. These starting materials have no ^{15}N–^{13}C bonds and no DCP difference signal. If a DCP difference signal appears after reaction, it indicates the formation of new N–C bonds since one-fourth of such bonds will be ^{15}N–^{13}C bonds. For this case, the Matthews reaction series may be summarized as



(VI)

In other words we now have an amide nitrogen with a DCP difference signal and hence a peptide nitrogen. The chemistry of reactions III–V can produce no DCP difference signal.

The second strategy involves a reaction between $H^{13}C^{15}N$ and $H^{12}C^{14}N$ in varying proportions. All the ^{15}N atoms enter into the reaction bonded to a single ^{13}C atom. If that bond remains intact and no new N–C bonds are formed, then the *rate* at which the DCP difference signal builds up during the ^{15}N to ^{13}C cross-polarization step will be independent of dilution by unlabeled HCN. However, if new ^{15}N–^{13}C bonds are formed, the participating ^{15}N atoms will acquire a second bond to a ^{13}C atom part of the time and the *rate* of DCP transfer will be increased for that kind of ^{15}N functionality (as compared with infinite dilution). An increasing transfer rate with increasing label concentration supports the formation of new ^{15}N–^{13}C bonds (see Analysis of DCP Transfer Kinetics section). Alternatively, this strategy can be used at a single (perhaps infinite) dilution. We expect only two chemical species reporting at 95 ppm. If they incorporate the original label pair into different kinds of ^{15}N–^{13}C bonds, which will have different drain rates, a study of the drain kinetics can tell as how much of each bond is present.

There is another way for the transfer rate to become concentration dependent in this combined-label strategy: if the labels scramble during reaction. This will be distinguishable from new bond formation in two ways. First, all nitrogens will be similarly affected whether or not they are capable of forming new N–C bonds. Second, the overall DCP transfer rates will decrease as dilution is increased, approaching proportionality as scrambling becomes complete. The question of scrambling will have to be addressed before either the double-label or separated-label experiments can be interpreted since both (in their simplest form) assume it is absent.

Experiments

Dry hydrogen cyanide gas was prepared by adding dry sulfuric acid[24] to sodium cyanide containing the appropriate mixture of labels. The hydrogen cyanide was then distilled under vacuum into a liquid nitrogen cooled flask containing a small amount of triethylamine (about 4% of the weight of HCN). This flask was sealed and allowed to stand at room temperature for 3 days. The HCN polymer was then removed under a nitrogen atmosphere. Hydrolysis was achieved by stirring the HCN polymer with cold water at room temperature for the desired number of days. Water was removed by lyophilization, after first separating the soluble and insoluble fractions (if desired).

Figure 1 gives the pulse sequences used to obtain both the CPMAS ^{15}N NMR spectra and DCP difference spectra of the labeled HCN polymer.[7,9,12] The first contact between ^{1}H and ^{15}N was for 5 ms (at 35 kHz), which is long enough to ensure representative intensities for all nitrogens. The hold and drain sequences are implemented in alternating 100-scan blocks which are accumulated separately in the signal-averaging computer. The hold sequence gives the normal CPMAS ^{15}N NMR spectrum and the difference between the two sequences the DCP difference spectrum. It is necessary to alternate hold and drain experiments in this way to prevent drifts in the spectrometer from producing a spurious DCP difference.

Experimental details of the 200-MHz (for ^{1}H) DCP spectrometer are given elsewhere.[12,25] The ^{15}N–^{13}C cross-polarization rate is a complicated function of the ^{15}N and ^{13}C radio-frequency fields as well as the spinning frequency.[9,26] The experiments presented

below were all performed at a spinning frequency of 3.205 kHz with ^{15}N and ^{13}C radio-frequency fields of 35 and 38 kHz, respectively. This corresponds to a maximum in the transfer rate. The transfer rate is sufficiently dependent on these parameters that it is necessary to regulate both the spinning speed and radio-frequency fields with electronic feed-back loops to do quantitative work.[25] The double-bearing sample spinner speed is monitored optically and controlled to ±1 Hz by means of a voltage-variable valve in the driving gas line. The radio-frequency fields are sensed in the probe by means of a loosely coupled pick-up coil and controlled to ±1% by means of a double-balanced mixer in each of the transmitter inputs. The scheme of four pulses for total suppression of sidebands (TOSS)[27] pictured in Figure 1 must be synchronized to the rotor period, so that its operation also improves with close control of the rotor speed.

Analysis of DCP Transfer Kinetics

Consider a nitrogen with the potential to be bound to two carbons C_a and C_b. For a given hold time τ (see Figure 1), the signal *with* drain S may be subtracted from the signal *without* drain S_0 to obtain the DCP difference signal $\Delta S = S_0 - S$ for any value of the chemical shift. If we assume that each ^{15}N atom has the probability p_a of having C_a be a ^{13}C atom (with drain rate T_a^{-1}) and the probability p_b of having C_b be a ^{13}C atom (with drain rate T_b^{-1}), then ΔS will be given by[9,11]

$$\Delta S/S_0 = p_a R_a + p_b R_b - p_a p_b R_a R_b \quad (1)$$

where

$$R_a = 1 - e^{-\tau/T_a} \qquad R_b = 1 - e^{-\tau/T_b} \quad (2)$$

We have assumed that drain rates are additive when both ^{13}C labels are present.

The signal at 95 ppm potentially is the sum of signals from two kinds of nitrogen. Suppose that a fraction P of this signal arises from >CH–^{15}NH–CO– nitrogens (peptides) and a fraction $(1 - P)$ of the signal arises from $^{15}NH_2$–CO–nitrogens (primary amides). Equation 1 must then be replaced by a weighted average

$$\Delta S/S_0 = P(p_a R_a + p_b R_b - p_a p_b R_a R_b) + (1 - P)p_a' R_a \quad (3)$$

We assign the subscript a to the N–CO bond and the subscript b to the N–CH bond. We expect the same value of T_a for both the peptide and primary amide bonds, but it is necessary to use p_a' for the probability that each primary amide ^{15}N is bonded to a ^{13}C atom. There is no p_b' since there is no N–CH bond in the primary amide. This analysis is going to ignore the natural-abundance ^{15}N signal since it is so small.

The first of our labeling strategies puts the ^{15}N and ^{13}C labels on separate HCN molecules. In this case (referring to reactions VI), $p_b = f_0$ (=0.01) the ^{13}C natural abundance in the ^{15}N-labeled HCN; and $p_a = fD + f_0(1 - D)$, where f (=0.99) represents the level of enrichment in the ^{13}C-labeled HCN and D represents the ^{13}C dilution factor by HCN molecules not enriched in ^{13}C (D = 0.5 for the equimolar mixture we have used). For the primary amides $p_a' = f_0$, and eq 3 becomes

$$\Delta S/S_0 = P\{[fD + f_0(1 - D)]R_a + f_0 R_b - f_0[fD + f_0(1 - D)]R_a R_b\} + (1 - P)f_0 R_a \quad (4)$$

Neglecting the terms in f_0 and rearranging, we obtain

$$P = \frac{\Delta S/S_0}{fD(1 - e^{-\tau/T_a})} \quad (5)$$

where we need only a rough idea of T_a to estimate the fraction of the signal arising from peptide nitrogens.

The second labeling strategy puts both labels on the same HCN molecule and varies the dilution factor. The only changes are $p_b = f = p_a'$. Neglecting terms in f_0, we obtain from eq 3

$$\Delta S/S_0 = P(fDR_a + fR_b - f^2 DR_a R_b) + (1 - P)fR_a \quad (6)$$

which may also be solved for P. Another way to use this relationship and also observe the interplay of the various parameters is to consider the case of small τ

$$(\Delta S/S_0)/f \sim \tau\left[\frac{1 - P(1 - D)}{T_a} + \frac{P}{T_b}\right] \quad (7)$$

Near the origin of a log $[1 - (\Delta S/S_0)/f]$ vs. τ plot, we see that

$$1 - (\Delta S/S_0)/f \sim \exp\left\{-\tau\left[\frac{1 - P(1 - D)}{T_a} + \frac{P}{T_b}\right]\right\} \quad (8)$$

Thus the slope of such a plot near $\tau = 0$ as a function of D will yield a value of P so long as we have reliable values of T_a and T_b from some other source.

Clearly the first (separated-label) strategy represents a more effective way to determine P since any DCP difference signal arises directly from the formation of N–CO bonds. The second (combined-label) strategy is basically a difference method requiring a *change* in drain rate, and in the $(HCN)_x$ system is therefore inherently less sensitive (and more work). However, the combined-label strategy, with its large DCP difference signal, does provide a way to check for label scrambling. Thus it complements the first method.

The above analysis assumes that the new N–C bond formed in the peptide-producing reaction is the N–CO bond because that is expected on chemical grounds.[13-16] That is not a limitation of the method. If part of the peptide linkages should form by means of a new N–CH bond, instead, then their contribution would have to be added to eq 3. The double-label probabilities for this case for each labeling strategy can then be obtained by applying the same kind of reasoning as used above. Since the DCP data do not support this alternative as a major factor, we have not bothered to give the actual (more complicated) equations.

Results

Single- and double-cross-polarization ^{15}N NMR spectra of the reaction products of an equimolar mixture of natural abundance HCN and $H^{13}C^{15}N$ (99% isotopic enrichment of both carbon and nitrogen) are shown in Figure 2. Nitrogen appears in a wide distribution of functionality.[6] Before hydrolysis, ^{15}N label appears in amines (40 ppm), olefinic and aromatic nitrogen (160–220 ppm), as well as nitrile nitrogen (240 ppm; Figure 2, left). After exposure to water under mild conditions, all sharp ^{15}N resonances (which include signals from diaminomaleonitrile) have disappeared from the spectrum of the insoluble fraction (Figure 2, right). In addition, the shape of the broad ^{15}N resonance has changed appreciably with, for example, sizeable new intensity shifted to near 95 ppm, the region associated exclusively with amide nitrogens. Both before and after exposure to water, a strong DCP difference signal is observed from the HCN reaction products, with a shape similar to that of the full spectrum. Qualitatively, this result indicates the absence of significant isotopic scrambling of one nitrogen chemical type relative to another.

The arrows in Figure 2 indicate points in the spectra where, as shown in Figure 3, matched spin-lock ^{15}N–^{13}C cross-polarization transfer rates were determined as a function of isotopic composition of the HCN reaction mixture as suggested by eq 8. This experiment tests quantitatively for the integrity of labeled C–N bonds. The sharp lines at 240 and 40 ppm, respectively, in the spectrum of the unhydrolyzed material show no dependence on the isotopic composition of the reaction mixture. The

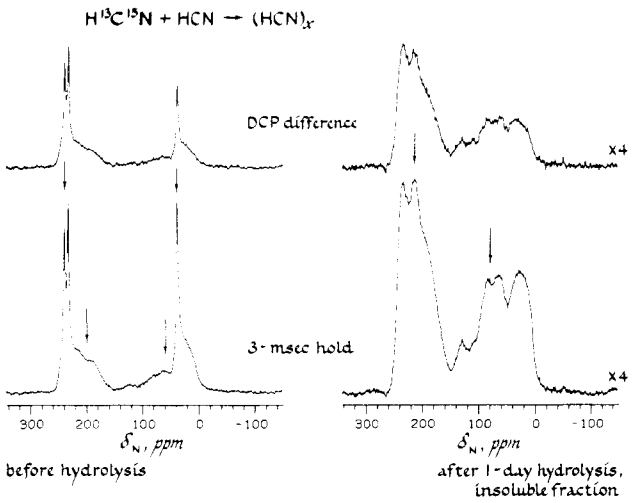


Figure 2. ^{15}N NMR (20.3 MHz) spectra of the dried reaction products of a 1:1 mixture of $H^{13}C^{15}N$ and HCN. The spectrum of the total solids before hydrolysis appears at the lower left of the figure. A ^{15}N spin-lock hold of 3 ms was used in obtaining this spectrum. The corresponding double-cross-polarization difference spectrum is also shown (upper left). Analogous ^{15}N NMR spectra of the water-insoluble fraction after a 1-day hydrolysis are shown at the right of the figure. Arrows mark the points in the spectra where carbon–nitrogen spin-lock transfer rate determinations are plotted in Figure 3.

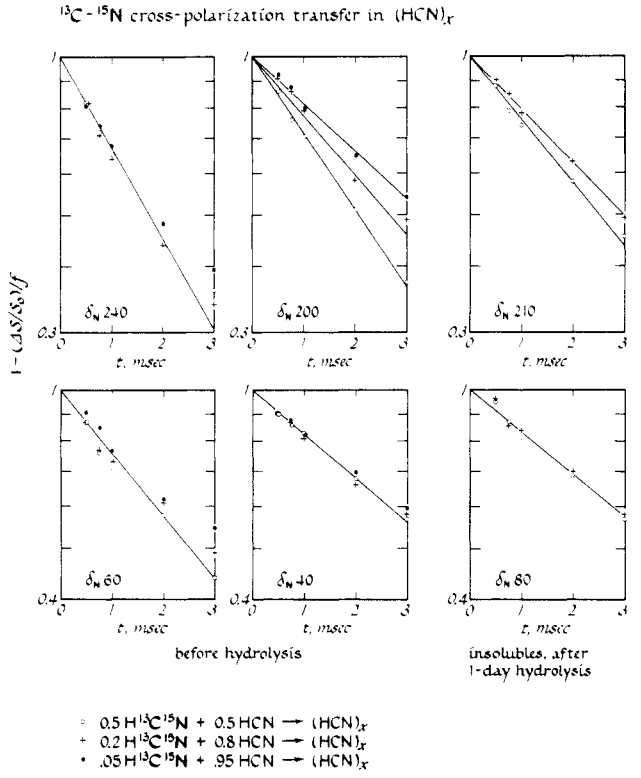


Figure 3. Determination of the carbon–nitrogen matched spin-lock cross-polarization transfer rate at various points in $(HCN)_x$ spectra as a function of the isotopic composition of the reaction mixture (compare Figure 2).

line at 60 ppm shows a weak dependence, while that at 200 ppm shows a significant dependence, with the transfer rate decreasing by about a factor of 2 for an increase in the ratio of concentration of HCN to $H^{13}C^{15}N$ of just under 20. The insoluble fraction of the hydrolyzed material also has ^{15}N lines with measurable dependencies of the transfer rate on isotopic composition.

Since some C≡N and C—N functionalities are observed with no measurable dependence of transfer rate on isotopic composition, the presence of scrambling during polymerization seems unlikely. That is, there is no evidence for bond breaking, isotopic exchange with an unreacted label pool, and bond reformation. The observed rate dependence on isotopic composition therefore indicates bond formation alone. This means that the $(HCN)_x$ reaction products contain nitrogens with chemically new C–N bonds. The absence of inherently broad lines, due to ^{15}N–^{15}N dipolar coupling resistant to magic-angle spinning, rules out the formation of new N–N bonds. (The observed CPMAS spectrum is broad, but the many inflections show the presence of a distribution of sharp lines.)

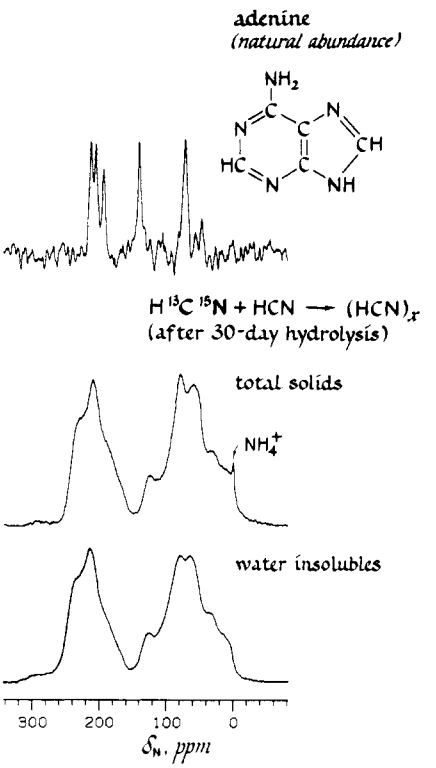


Figure 4. ^{15}N NMR (20.3 MHz) spectra of the dried reaction products of a 1:1 mixture of $H^{13}C^{15}N$ and HCN after a 30-day hydrolysis (middle and bottom). The spectrum shown at the top of the figure is that of adenine at natural abundance. Separate peaks for all five nitrogens are observed in the solid state, with that at 70 ppm due to NH_2, that at 140 ppm due to NH, and the three peaks near 210 ppm due to the three nonprotonated aromatic nitrogens. The external reference is solid ammonium sulfate (0 ppm). All three spectra were obtained without the use of an ^{15}N hold and drain delay but with the use of TOSS.

The appearance of the ^{15}N NMR spectrum of $(HCN)_x$ changes with continued exposure to water. In particular, there is a shift in relative intensity from the low-field 200-ppm region to the midfield 90-ppm region (compare Figure 2, bottom right, and Figure 4, bottom). The only sharp line remaining in the spectrum of the total solids is due to $^{15}NH_4^+$. (Labeled ammonia is only observed by NMR after exposure of $(HCN)_x$ to water.) Over 90% of all the nitrogen in the HCN reaction products is found in the water-insoluble fraction for this polymerization. The variation in types of nitrogen functionality found in this fraction, after extensive exposure to water, is still greater than that found in, for example, a typical purine like adenine (Figure 4). The strong DCP difference signal observed in general for all the nitrogens of the insoluble fraction of the 30-day-hydrolyzed material and in particular for the resonance at 95 ppm (Figure 5), indicates the long-term stability of labeled pairs to mild water conditions.

The results of the separated, double-label experiment are illustrated in Figure 6. In this experiment, the ^{13}C

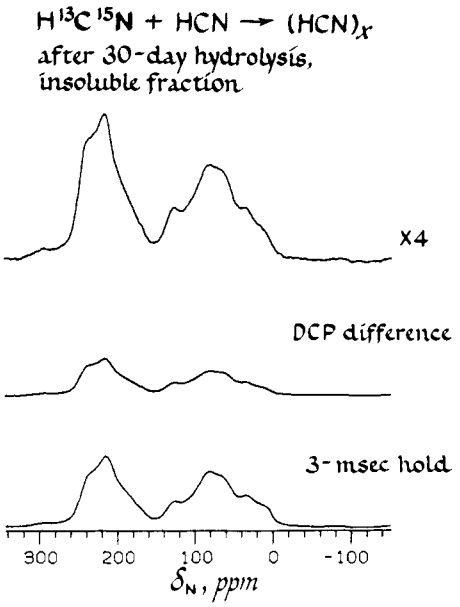


Figure 5. ^{15}N NMR (20.3 MHz) spectra of the water-insoluble fraction of the reaction products of a 1:1 mixture of $H^{13}C^{15}N$ and HCN after a 30-day hydrolysis. The full spectrum following a 3-ms ^{15}N spin-lock delay is shown at the bottom, and the corresponding double-cross-polarization difference spectrum at the top of the figure.

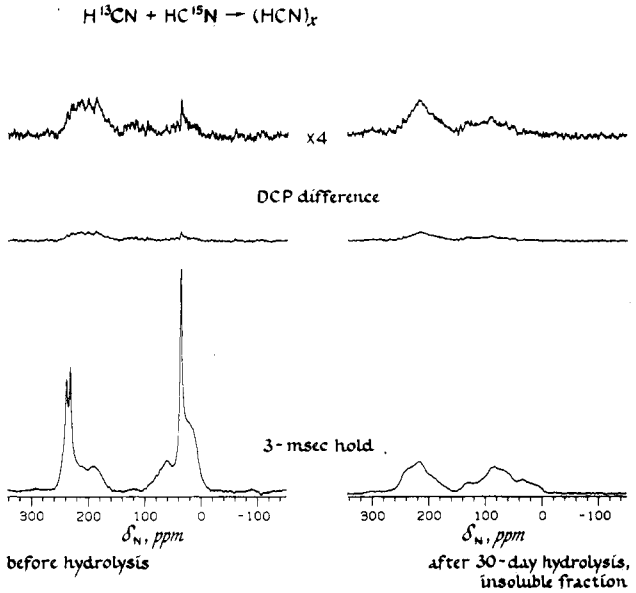


Figure 6. ^{15}N NMR (20.3 MHz) spectra of the dried reaction products of a 1:1 mixture of $H^{13}CN$ and $HC^{15}N$. The spectrum of the total solids before hydrolysis following a 3-ms ^{15}N spin-lock delay is shown at the bottom left, and the corresponding double-cross-polarization difference spectrum at the upper left of the figure. Analogous ^{15}N NMR spectra of the water-insoluble fraction after a 30-day hydrolysis are shown at the right of the figure. The single-cross spectra have been scaled so that the areas of the broad components are equal.

label is placed on one HCN molecule and the ^{15}N label on another. The observation of a DCP difference signal therefore can be associated immediately with nitrogens forming new bonds since scrambling has already been ruled out. (Figure 6 also supports the conclusion of negligible scrambling. The sharp line at 40 ppm in the HCN polymer before hydrolysis arises largely from the amino groups in diaminomaleonitrile. Without scrambling, it will give no DCP difference signal above that expected from natural abundance ^{13}C in the $HC^{15}N$. The DCP difference signal which is seen is only a few times the natural abundance level, indicating little scrambling.) Consistent with the

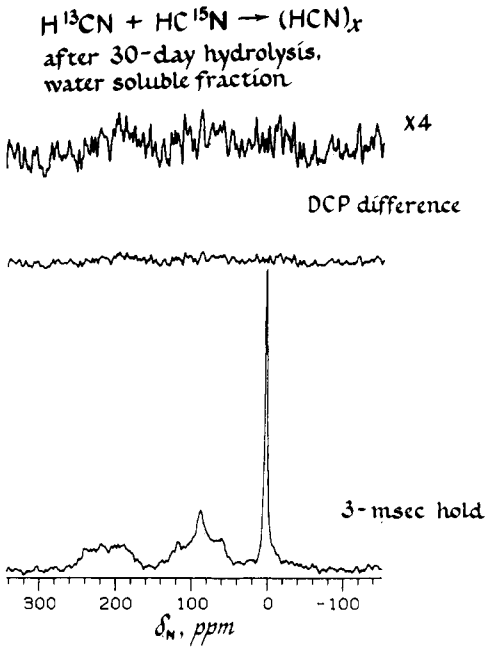


Figure 7. ^{15}N NMR (20.3 MHz) spectra of the water-soluble fraction of the reaction products of 1:1 mixture of $H^{13}CN$ and $HC^{15}N$ after a 30-day hydrolysis. The full spectrum following a 3-ms ^{15}N spin-lock delay is shown at the bottom, and the corresponding double-cross-polarization difference spectrum at the top of the figure.

results of the double-label dilution experiment of Figure 3, we observe strong DCP difference signals in the mixed-label HCN reaction products only near 200 ppm, for material both before and after hydrolysis. However, other DCP difference signals can be clearly seen, such as, for example, that at 40 ppm in the spectrum of the total solids before hydrolysis (Figure 6, left), as well as that at 95 ppm in the spectrum of the water-insoluble fraction (which contains 60% of the total weight) after 30-day hydrolysis (Figure 6, right). For this kind of system, the mixed-label technique is clearly better suited to the detection of smaller DCP difference signals than is the double-label dilution procedure of Figure 3. The latter procedure, however, is valuable in reaction systems where exchange, mixing, or competition with large unlabeled pools occurs.

There is no DCP difference observed for any of the nitrogens of the water-soluble fraction of the mixed-label reaction products after 30-day hydrolysis (Figure 7), although the limited sample size would make weak signals difficult to detect. This is consistent with the fact that previous efforts to detect heteropolypeptides in the water-soluble fraction after hydrolysis have been inconclusive.[22] [Note added in proof: The separated double-label experiment has been repeated using 5 times as much labeled material. The resulting spectra are much improved in signal-to-noise ratio compared to those of Figures 6 and 7, but can be interpreted the same way.]

Discussion

^{15}N–^{13}C Transfer Rates. One of the objectives of this study was to look for evidence of heteropolypeptides in HCN polymer. It is clear in Figures 1, 3, 5, and 6 that the spectra of the insoluble hydrolysate show appreciable absorption near 95 ppm. The presence of a DCP difference signal at that shift value in the mixed-label experiment (Figure 6, right) shows that part of those nitrogens must occur in the peptide linkage (cf. reaction VI). If we estimate that the DCP difference signal ΔS is about 20% of the 3-ms hold signal S_0 at 95 ppm, then $\Delta S/S_0 = 0.2$ for

τ = 3 ms. Taking D = 0.5, f = 0.99, and T_a = 4.4 ms (as determined for the amide nitrogen in solid asparagine),[9] we calculate $P \approx 0.8$. In other words, about $^4/_5$ of the intensity at 95 ppm represents nitrogens in the peptide linkage. (We have made an independent estimate of this quantity from the data in Figure 5, using eq 6. The results are consistent, giving $P \approx 1$. However, this approach depends too heavily on the values of T_a and T_b used and is too insensitive to P to be useful for more than a qualitative corroboration of the separated-label result.) It is not possible to say how long a run of heteropolypeptide is present; but, if one peptide bond is formed, more than one seems likely. The fact that this is the insoluble hydrolysate suggests relatively high molecular weights.

We have tried to fit the DCP difference data in Figures 5 and 6 assuming that it is the N–CH bond that forms when the peptide linkage forms instead of the N–CO bond as indicated by reactions I and II. The analysis yields different, nonphysical results for these two different situations. This is because T_b (as determined in solid, double-labeled glycine)[9] is much longer than T_a. There is too much DCP signal in the separated-label case and not enough in the combined-label case to be fit separately or together by a reasonable value of P. It is not possible to rule out a small amount of this alternative chemistry taking place, but it cannot be a major component.

Absence of Amide Nitrogen Formation by Tautomeric Exchange in the Separated Label Experiment. HCN can conceivably undergo aminolysis to formamidine, $HCN + NH_3 \rightarrow H_2N{-}CH{=}NH$, with similar reactions possible for various other –CN functionalities. We know free ammonia is present after hydrolysis from inspection of Figure 4. If this ammonia is labeled, then tautomerism could lead to

$$H_2{}^{14}N{-}{}^{13}CH{=}{}^{15}NH \rightleftharpoons H{}^{14}N{=}{}^{13}CH{-}{}^{15}NH_2$$
$$H{}^{14}N{=}{}^{13}CH{-}{}^{15}NH_2 + H_2O \rightarrow {-}{}^{13}C(=O){-}{}^{15}NH_2 \qquad \text{(VII)}$$

That is, double label would appear in a primary amide functionality (giving rise to a DCP difference signal at 95 ppm) by chemistry other than that represented by reaction VI for separated labels.

We can rule this possibility out, however. If all the signal intensity at 95 ppm were due to primary amide, the DCP difference signal ($\Delta S/S_0$) at 95 ppm in the absence of scrambling would be 0.49 for the combined-label case (Figure 5) and zero for the separated-label case (Figure 6). This calculation assumes T_a = 4.4 ms and neglects natural abundance signals. If scrambling were complete, both cases would yield the *same* value of $\Delta S/S_0$ = 0.24. The actual values, taken from Figures 5 and 6, are 0.4 and 0.2, respectively. This disparity rules out scrambling as a major factor since partial scrambling would cause these two values to approach the common value (0.24) at the same rate.

Summary of Results for HCN Polymerization. We have shown by the use of chemical-bond labeling and DCP ^{15}N NMR that a small but significant amount of heteropolypeptide is formed when hydrogen cyanide is allowed to polymerize in a basic environment and then is hydrolyzed. The spectral intensity for peptide nitrogens (near 95 ppm) does not represent a large fraction of the total intensity in the ^{15}N spectrum of the insoluble hydrolysate. It seems likely from the complexity of this spectrum that many other structures must also be present, particularly aromatic nitrogen compounds such as purines, the cross-linked ladder structures of Volker,[5] and the polymeric extensions of diaminomaleonitrile proposed by Ferris.[22] The ability of ^{15}N NMR to sort out useful information about peptide formation from such a complex mixture is due to its dealing with nitrogen functionality (as resolved on an NMR chemical shift scale), rather than with specific nitrogen compounds. Such compounds are chemical entities whose resolution and identification require elaborate and delicate separation strategies.

Registry No. HCN homopolymer, 26746-21-4.

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