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The Radiolysis of Aqueous Ammonium Cyanide: Compounds of Interest to Chemical Evolution Studies

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Summary. Oxygen-free aqueous solutions of NH_4CN (0.1 M, pH 9) were exposed to gamma rays from a ^{60}Co source, the mixture of nonvolatile products was fractionated, and the fractions were analyzed. The procedures were chosen to make effective investigations of radiolytic products, and to minimize the contributions of chemical changes which are known to occur in aqueous solution in the absence of ionizing radiation. It has been found that the main constituents are: urea, 25.9%; an oligomer, very likely oligoimine (18.4%); and several fractions (about 50%) which release amino acids on hydrolysis. These fractions differ considerably, as shown by amino acid assay, enzymatic digestion, IR spectra, and biuret reaction. All these tests were found to be positive for two fractions; in two further fractions the enzymatic cleavage was absent, but other tests were positive. Negative enzymatic and biuret tests, and no bands characteristic of amide or peptide, were found for a fraction whose hydrolysate consisted of 55% glycine. Although most of the isolated materials were found to be composite, the results of the analyses were sufficient for getting a reliable over-all picture of the chemical action of the ionizing radiation. The role of free radicals in reactions leading to the formations of radiolytic products was considered.

Key Words: Aqueous solutions – Cyanides – Ionizing radiation – Fractionation of products – Chemical evolution – Additional oligomerization

Introduction

A recent survey of the radiation chemistry of aqueous solutions of simple cyano compounds, and the examination of the role of ionizing radiation as one of the energy sources on the early Earth, suggest that radiation-induced chemical processes in an aqueous environment could be more important for prebiotic chemistry than presently realized (Draganić and Draganić, 1980). Some findings concerning the radiolysis of aqueous ammonium cyanide (0.1 M, O_2-free, pH 9) seem to be of particular interest.

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Examination of mixtures of radiolytic products suggested the presence of oligomers that have (or whose fragments have) peptidic properties. Supporting evidence was obtained by the biuret test, by measurements of amino acid contents in hydrolysates, by amino acid identifications using GC-MS analysis, and by recording and analyzing the IR spectra (Draganić et al., 1977a, 1977b, 1977c, and 1977d). Experiments were made with mixtures of radiolytic products formed at various radiation doses, and the phenomena observed could be correlated with amounts of ionizing energy absorbed. The purpose of this work was to fractionate a mixture of the nonvolatile products formed at a larger dose (mainly about 18 Mrad), where the conversion of cyanide ion is about 75%, and to analyze the fractionated material.

Experimental

Preparation of Samples

Solutions. The purification of water and the cleaning of glassware were carried out by the standard procedures used in the radiation chemistry of aqueous solutions. Hydrogen cyanide was freshly prepared from sodium cyanide and de-aerated sulfuric acid. By a simple setup, in an argon atmosphere, gaseous HCN was introduced into de-aerated water. Ammonium cyanide samples were prepared by mixing fresh aqueous solutions of HCN and ammonia. The syringe technique was used for sample preparation and irradiations (Draganić et al., 1976b; 1977a).

Materials. Sephadex and blue Dextran were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden), Dowex from Fluka (Basel, Switzerland), and CM-52 and DE-52 Cellulose from Whatman (Madstone, England). Sephadex G-15 was treated with HCl (Goodson and Di Stefano, 1969; Goodson et al., 1971). Pig insulin was supplied by Novo (Copenhagen, Denmark); hexaglycine, tetraglycine, glycylsarcosine, and glutathione were Sigma products (Saint Louis, Mi., U.S.A.). The urea enzymatic colorimetric test kit was obtained from Boehringer (Mannheim, West Germany), aminopeptidase M from Röhm (Munich, West Germany), and pronase B grade (lot N^{o} 802684) from Calbiochem (Lucerne, Switzerland). All other chemicals were of the highest purity available. The solvents were purified by standard procedures.

Irradiations. The ^{60}Co γ-radiation unit at Vinča was used at dose rates of about 3.10^{17} eV g^{-1} min^{-1}. Absorbed doses varied between 9.10^{20} eV g^{-1} and 13.10^{20} eV g^{-1} (i.e., 15–20 Mrads). Irradiations were made at room temperature.

Irradiated Samples. After irradiation the solution was evaporated to dryness, under a reduced pressure, at 45°C. The mixture of nonvolatile radiolytic products appears as a dark-brown, oily, residue which was dried over P_2O_5, weighed, and stored under refrigeration.

Before analysis the residue was dissolved in water, 400–500 mg per 1 ml of H_2O, and the pH was adjusted by addition of acetic acid to pH 4–5. The solution was kept until the pattern of ninhydrin positive substances on an amino acid analyser remained constant, usually 20 hours. This mild treatment, which could not affect the peptide bond, was chosen in order to facilitate a subsequent fractionation of the mixture. A small amount of the dark brown, insoluble precipitate (ca. 0.4%), which was formed during sample conditioning, was removed and was not further examined. It is worth

noticing that similar precipitates also appeared in aqueous solutions of acid and basic fractions when they were kept at ambient temperature for longer periods of time. Besides that, the formation of ammonia was observed in aqueous solutions of almost all fractions. This is why the fractionated material was usually kept dry refrigerated, and was dissolved only before the analysis.

Method and Analyses

Spectrometry. UV spectra were recorded on Varian 634, and IR spectra on a Beckman 4220 or a Perkin Elmer 457 instrument. For IR spectra the sample was dissolved in water and then lyophilized in the presence of 300 mg of KBr; a quantity of up to 150 mg of the lyophilized material was used for a pressed pellet. Graphical resolution was applied in the analysis of recorded spectra (Vandeginste and De Galan, 1975). To facilitate a comparison of characteristic frequencies, registered under different experimental conditions, the relative intensities of absorption bands (maximum peak height × half width) were divided by relative intensity of carbonyl absorption. When some IR measurements had to be performed on deuterated samples, the dry residue was dissolved in 1 ml of heavy water, in an argon atmosphere, and kept in a closed glass vessel for about 40 h at 80°C.

High-Voltage Paper Electrophoresis (HVE). A Pherograph Mini-68 instrument, at 2200 V and 12°C, was used with Whatman 1 or 3 MM papers. The following buffers were used: for pH 2, 8% acetic acid and 2% formic acid (AF); for pH 5.2, 2% pyridine and 1% acetic acid (PA), (Ambler, 1963; Bailey, 1967).

Paper Chromatography. The ascending technique on Whatman 1 or 3 MM papers was used with the following solvent systems: n-propanol/water, 7:3 (PW), n-butanol/acetic acid/water, 4:1:5 (BAW), and n-propanol/ammonia (25%), 6:4 (PAm).

Visualization of Spots. It was made by exposing papers to 254 nm or 366 nm light and by staining with the following reagents: ninhydrin/Cd(II),(Heathcote and Haworth, 1964); chlorine/iodine, (Kasper, 1970); and dimethylbenzaldehyde/HCl, (DBA), (Lee, 1972).

Biuret Method. The modified method (Ellman, 1962) was used: it is based on absorbance changes in the UV range of the alkaline copper (II) solution. For the calculation of peptide bonds we have used 800 M^{-1} cm^{-1} for molar absorptivity at the peak maximum of the measured complex.

Determination of Amino Acids. The standard procedure for protein hydrolysates was used with the amino acid analysers (Beckman Unichrom and Beckman 120B, Palo Alto, Ca., U.S.A.). Usually 1–2 mg of samples were hydrolyzed with 1–2 ml of 6N HCl in sealed vials at 110°C for 20 h.

Amino acids were assigned on the basis of their retention times on the amino acid analyzer. Comparison was made with the standard set of amino acids from protein hydrolysates and with some nonprotein amino acid references. Also, some nonprotein amino acids were assigned by fitting the peak positions to those known in the literature (Hamilton, 1963; Shapshak and Ohaji, 1972). Supporting evidence for the

presence of aspartic acid, β-CH_3-aspartic acid, sarcosine, glycine, alanine, and β-alanine was obtained by GC-MS determinations in our previous work with hydrolysates of a mixture of radiolytic products in the same system (Draganić et al., 1977b, 1977c). As distinct from our previous study, we have used here up to 100 times more concentrated samples for the analysis of amino acids. New peaks appeared and were assigned to lysine, histidine, and α-NH_2-butyric acid. Their identification is tentative, since structurally similar compounds may exhibit similar chromatographic behavior under our working conditions. The positive Pauly test, and the IR spectrum gave some supporting evidence for histidine and lysine respectively. Several peaks appearing before aspartic acid remained unassigned.

Urea and Ammonia. A Boehringer test kit was used as described by the manufacturer.

Attempts to Estimate the Molecular Weights of Fractions. We have tried to use for this purpose Sephadex G-25, G-15, and G-15-HCl treated. Natural peptides behaved normally under our working conditions as judged by a comparison with test molecules (Goodson and Di Stefano, 1969; Goodson et al., 1971). A strong retardation was observed in radiolytically produced materials. Similar behavior was already reported for base catalyzed oligomers of NH_4CN (Matthews and Moser, 1967; Ferris et al., 1973).

Enzymatic Digestion. The procedure used for aminopeptidase M was essentially the same as that due to Light (1972): 0.2 ml of the enzyme solution (1 mg/ml) in a 0.1 M phosphate buffer (pH 7) was added to 0.2 ml of sample solution (20–80 mg/ml). The samples were incubated at 37°C for 20 h. The enzymatic digestion with pronase was performed in a 0.15 M borate buffer containing 0.01 M $CaCl_2$ (Löfquist and Sjöberg, 1971). The samples were incubated at 37°C for 20 or 48 h. Blanks containing the pure sample as well as the pure enzyme were always run in parallel. For comparison, the digestion of hexaglycine was made, too. The digestion was followed by an increase in the color yield with the ninhydrin reagent (Cocking and Yemm, 1954), by a comparison of the digested and blank samples on HV electrophoresis (buffer AF), by paper chromatography (solvent PAm), and/or by amino acid analyser measurement.

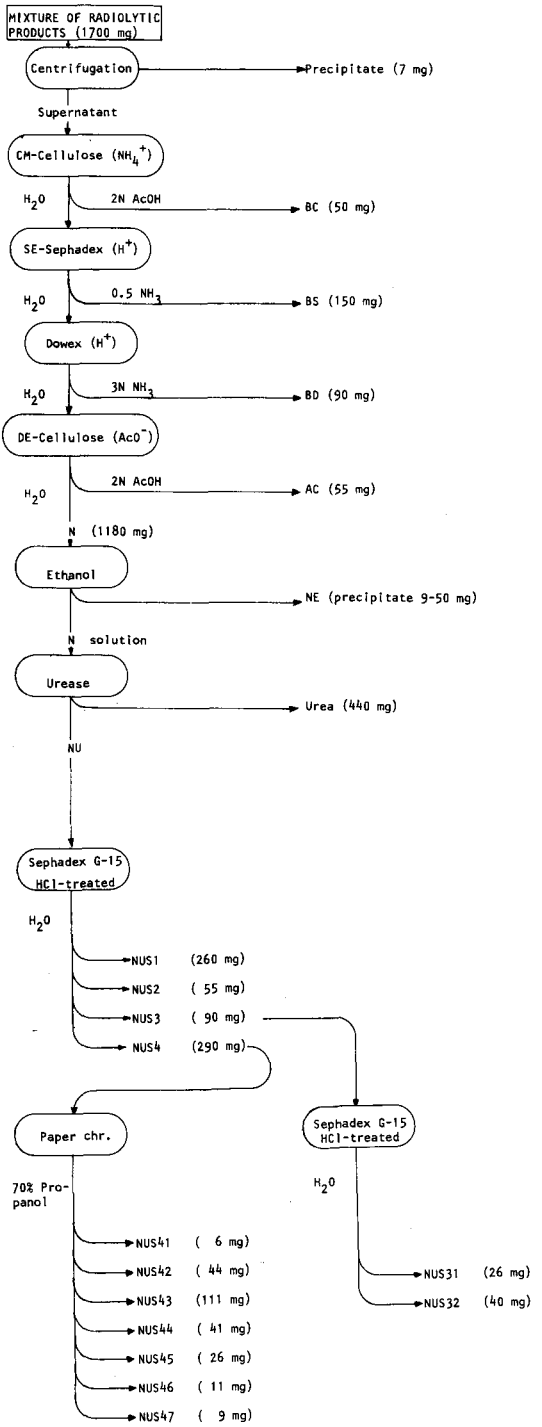
The digestion with urease was performed by adding 1.5 ml of a urease solution to 4–5 ml of the sample solution (ca. 200 mg/ml) and by keeping the resulting mixture at ambient temperature for 20 h.

Fractionation of a Mixture of Radiolytic Products

General

Preliminary examinations showed that procedures used for the fractionation of a mixture of products from a base-catalyzed oligomerisation of NH_4CN can not be applied to a mixture of radiolytically produced compounds. A direct application of a radiolytic mixture to a Sephadex column, similar to the procedure due to Matthews and Moser (1967), and Ferris et al. (1973), revealed only one broad peak, intensely UV absorbing, which corresponded to the ninhydrin positive peak obtained after the alkaline hydrolysis of the sample. A direct application to a Dowex column (Ferris et al., 1974) resulted in a strong, irreversible, retardation of a significant amount of

Chart 1. Fraction of mixture of radiolytic products. Details on labelling of fractions are given in the text



the analyzed material. Standard procedures for the fractionation of materials of biological origin (Schroeder, 1967) also could not be used because of difficulties in removing the buffers which had to be introduced in the course of separation. This is why a specific procedure was devised, which is based on the application of weak hydrophylic ion-exchangers that facilitate the elution of charged material under milder conditions. In this way we have isolated three fractions with the basic character (BC, BS and BD), one acidic (AC), and several neutral fractions. Chart 1 outlines the procedure adopted for the fractionation.

The fractions in Chart 1 are labelled as follows. The first letter specifies the nature of the fraction: B = basic, A = acidic, N = neutral. The second letter specifies the type of column used for the isolation of the fraction: C = cellulose, S = sephadex, D = Dowex, or any special treatments such as E = precipitation with ethanol and U = digestion with urease. In the three-letter labels the order of the second and the third letter corresponds to the sequence of operations, the letter codes being the same as above. Numerals are used to label different fractions obtained by the elution with the same solvent or a solvent mixture. In the two-digit labels the first digit specifies the fraction number from the preceeding operation.

Details of the Procedure Given in Chart 1

Column media were prepared according to manufacturers' directions, or common procedures. The elution was carried out until the absorbance at 220 or 230 nm decreased to 0.1–0.2. Bands from preparative chromatograms were eluted with distilled water. All solid substances were isolated by concentration of the solutions on a rotary evaporator at 45°C and at reduced pressure, followed by lyophylization.

In routine work, the starting material consisted of 600 ml of O_2-free, aqueous ammonium cyanide to which a 18 Mrad dose was given. This gave about 1700 mg of dry residue containing a mixture of nonvolatile radiolytic products.

The dry residue was dissolved in 4–5 ml of water, undissolved material was centrifuged off, the pH was adjusted to 9, and the sample was applied to a CM-Cellulose column in the NH_4^+ form (2.5 × 3.5 cm). The column was eluted first with 200 ml of water and then with 100 ml of 2N acetic acid. The acetic acid eluate yields 50 mg of a strong basic fraction (BC) on lyophylization. The water eluate was concentrated, adjusted to pH 2–3, and the solution applied to a SE-Sephadex column in the H^+ form (2.5 × 13 cm). The column was eluted first with 500 ml of water, then with 100 ml of 0.5N NH_3. The ammonia eluate yields on lyophylization 150 mg of basic fraction BS. The water eluate was concentrated, adjusted to pH 2, and applied to a Dowex 50 × 8 column in the H^+ form (2.5 × 5 cm). The column was eluted with 500 ml of water and then with 200 ml of 3N NH_3. The ammonia eluate was lyophilized to yield 90 mg of the basic fraction BD. After concentration the pH was adjusted to 9 in the water eluate, which was then applied to a DE-cellulose column in the acetate form (2.5 × 5 cm). After elution with 500 ml of water 1180 mg of neutral fraction (N) are obtained. The subsequent elution with 100 ml of 2N acetic acid yields 55 mg of the acidic fraction (AC). On addition of absolute ethanol to concentrated aqueous solution of the fraction N, a white precipitate appears (NE). Depending on conditions its amount varies from 9 to 50 mg; it was centrifuged off. An ethanolic solution of N was evaporated to dryness, dissolved in water by taking 1 ml of H_2O per 200 mg

of sample, and digested with urease. The solution NU, urea-free, was then applied to a Sephadex G-15, HCl treated (1.5 X 80 cm). This was done by taking only one quarter of the solution, 1 ml, but the procedure was subsequently repeated three times and the corresponding fractions were pooled together. The fractionation of the neutral material on Sephadex gives the fractions NUS1, NUS2, NUS3, and NUS4. When the rechromatography was made under the same conditions, the fractions NUS2 and NUS4 remained unchanged; NUS3 was separated in NUS31 and NUS32.

A further fractionation of NUS4 was, however, achieved by means of paper chromatography in PW as the solvent.

Characterization of Fractionated Materials

We have analyzed the fractions, obtained by the procedure described above (Chart 1), by using methods which we have previously applied for the characterization of mixtures of products. The characterization of fractions of four different runs was performed. Each run concerns a fresh ammonium cyanide sample, its irradiation and fractionation. In one case the fractionation was carried out immediately after irradiation, while in other cases the dry residues containing a mixture of products were stored under refrigeration up to several months. No significant differences from batch to batch were noted.

Some general characteristics of the fractionated material are summarized in Table 1. The results of amino acid analyses are given in Table 2.

Table 3 shows the results of a graphical analysis of IR spectra recorded for various fractions.

The data presented in Tables 1–3 show the complexity of chemical changes induced by ionizing radiation in aqueous solutions of ammonium cyanide. Compared to the unfractionated mixture, some fractions appear impoverished or enriched in amino acids; they differ also by the abundance of their amino acid constituents. Differences in the physical appearance of fractionated materials should also be noted. An analysis of infra-red spectra offers some supplementary information consistent with other findings. Although most of the fractions appear as composite materials and further fractionation is desirable, these findings show that the present work on the isolation and characterization of radiolytic products represents a meaningful step towards a better insight into the mixture of radiolytic products. About 40% of the mixture are various fractions containing material with peptidic properties. Other constituents are: urea 25.9% and NUS1 16.4%; the hydrolysate of fraction NUS43 (8.2%) is very rich in glycine, but the search for peptide bonding gave clearly negative answers.

Table 1. Abundances of isolated fractions and their amino acid and peptide bond contents

Material	Amount	Amino acid content in hydrolysate		Estimate of peptide bond content	Remarks
	in % of the total weight of mixture	in μmols per g of mixture or fraction	in % of the total weight of fraction	in μmols per g of mixture or fraction	
Mixture	100[a]	1934	15[b]	2940	Brownish oil
Basic	17				
BC	2.9	361	5[c]	960	Brown oil
BS	8.8	2480	21[b]	5800	Brown powder
BD	5.3	1660	13.5[b]	1500	Light brown powder
Acidic					
AC	3.2	747	8[c]	1700	Light brown powder
Neutral	69	2353	19[b]	1740	Light yellow oil
NE ppt	0.6	1000	10[c]	2100	White powder
Urea[d]	25.9	— —	—	— —	—
NUS 1	16.4	trace	—	2000	Light yellow oil
NUS 2	3.4	900	9[b]	3200	Yellow oil
NUS 31	1.7	2258	23[b]	2100	White powder
NUS 32	2.6	1900	15[b]	2100	Yellow oil
NUS 4	18.4	6486	52[b]	1550	White powder
NUS 42	3.3	2530	30[c]	1300	Amorphous
NUS 43	8.2	9600	55[e]	trace	White powder
NUS 44	3.0	3500	20[e]	1400	Amorphous
NUS 45	3.0	700	8[c]	1200	Amorphous

[a] Total recovery (all fractions) was 89.2%
[b] Average molecular weight <MW> taken as 80
[c] <MW> taken as 100
[d] Determined after action of urease
[e] Calculated as bound Gly (MW = 75)

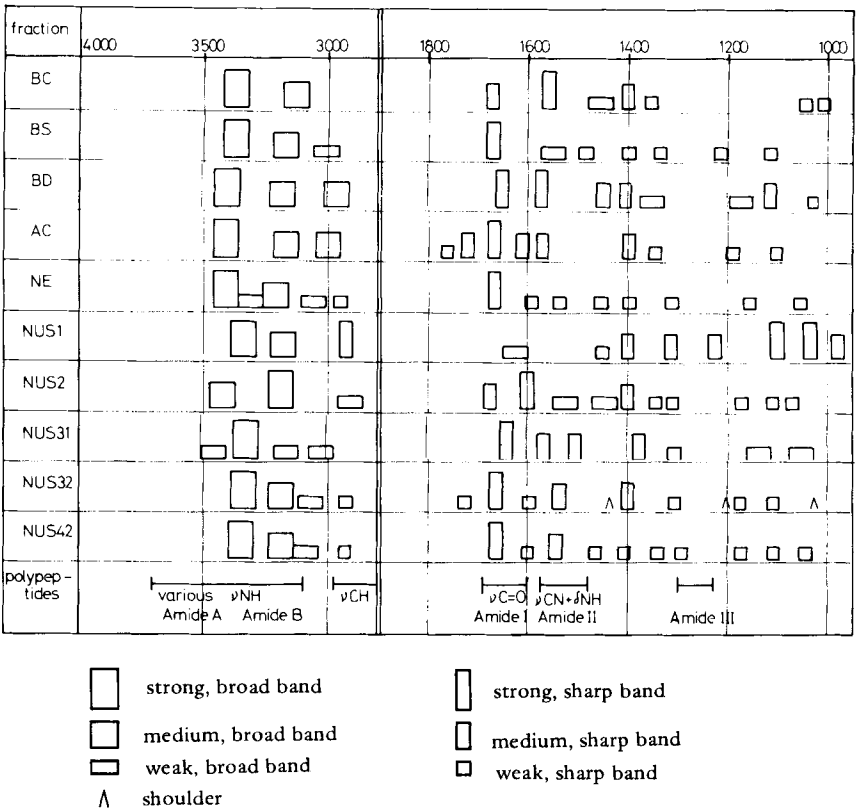
Table 2. Amino acid composition of isolated fractions in micromols per gram of the material

	Mixture	BC	BS	BD	AC	NE	NUS 31	NUS 4	NUS 42	NUS 43	NUS 44	NUS 45
Lysine	30	30	– –	– –	10	20	31	– –	– –	– –	– –	– –
Histidine	70	40	30	30	40	100	93	123	680	– –	– –	– –
Methylamine	[a]	(130)	(20)	(60)	(60)	– –	– –	– –	– –	– –	– –	– –
$A_{34,39}$[b]	– –	– –	– –	– –	– –	– –	217	– –	– –	– –	– –	– –
A_{43}	2	– –	20	40	23	– –	– –	– –	– –	– –	– –	– –
A_{44}	2	– –	20	– –	24	10	– –	– –	– –	– –	– –	– –
A_{46}	40	8	70	– –	50	30	310	10	140	– –	– –	– –
Aspartic Acid	100	20	– –	110	110	60	180	52	460	– –	– –	60
β-CH_3-Aspartic	[a]	– –	330	– –	2	3	– –	5	140	– –	– –	– –
Threonine	4	– –	20	– –	– –	10	124	– –	– –	– –	– –	– –
Serine	16	20	20	40	8	40	124	12	140	– –	– –	60
Sarcosine	14	– –	210	– –	70	10	– –	[a]	430	– –	– –	– –
Glutamic Acid	4	8	20	– –	10	10	37	12	– –	– –	– –	60
Citrulline	[a]	– –	– –	– –	– –	– –	– 75	– –	– –	– –	– –	– –
Glycine	1600	210	1700	1400	360	2000	980	6240	540	9600	3400	340
Alanine	45	21	40	40	40	50	64	32	– –	– –	– –	180
α-NH_2-Butyric	– –	4	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
β-Alanine	7	– –	– –	– –	– –	10	23	– –	– –	– –	– –	– –
Total	1934	361	2480	1660	747	2353	2258	6486	2530	9600	3400	700

[a] Trace amounts

[b] A_{34}, A_{39} ... A_{46}: unidentified peaks appearing before Aspartic Acid with various retention times

Table 3. Schematic Presentation of Resolved IR Spectra of Fractionated Materials



Details on Fractions

Fraction BC. The unhydrolyzed sample reveals on amino acid analysis only some unidentified peaks appearing after arginine. Its infra-red spectrum matches that of lysine dihydrochloride in many details such as the distribution of characteristic bands, and their relative intensities. The bands characteristic for all three groups ($-NH_3^+$, $-COO^-$, $-NH_2$) of the amino acid are present in the spectrum and suggest that lysine is C—C bonded at the end of the oligomer. Its amount should be considerably larger than that found in the hydrolysate (Table 2), which could be the case if it is not peptide bonded.

The analysis of the hydrolysate reveals, besides amino acids common to other fractions, the presence of a significant amount of CH_3NH_2. HVE shows that BC has a strong basic character. Enzymatic digestions with aminopeptidase M and pronase gave only marginal differences in comparison with blanks.

Fraction BS. This fraction is richer in amino acids than the original mixture, which is partly due to the presence of nonpeptidic amino acids, β-CH_3-aspartic acid, and sarco-

sine, as well as some unidentified products which appear regularly before aspartic acid in this and some other fractions.

Figure 1 summarizes the results of the enzymatic digestion of the fraction BS. The action of aminopeptidase M is clearly positive. Under the same conditions the blank with aminopeptidase M gives no positive reaction with ninhydrin, while a hexaglycine reference sample is completely digested. The action of pronase is weak.

The determination of amino acids in the material digested by aminopeptidase M shows that their concentrations are much lower than those measured in the hydrolysate after acid hydrolysis (Table 2): we found only 10% of sarcosine, 2% of glycine, and up to 50% for other amino acids. This finding points to the presence of only a limited number of free N-terminal amino acids. It is in agreement with an observation made earlier on the mixture of radiolytic products of the same system (Niketić, Buchart, Nebelin, unpublished results), when N-terminal amino acids were determined by phenylthiohydantoin degradation using the same technique as for natural peptides. PTH amino acids were identified by GC, MS, and by an amino acid analyser after acid hydrolysis. The total yield of N-terminal amino acids was estimated to be one thousandth of the amino acids present.

Fraction BD. Table 1 shows a good agreement between the total amino acid content in this fraction and the estimate of peptide bonds based on biuret method data.

The digestion with pronase is clearly positive in spite of some autodigestion of pronase (Fig. 2). It can be seen that after digestion some new peaks appear while some of those present in the blank disappear. A strong increase observed on new peaks of BD + pronase might be due to the release of N-terminals in the digested material. The difference in the patterns of the pronase blank and BD + pronase indicates also a

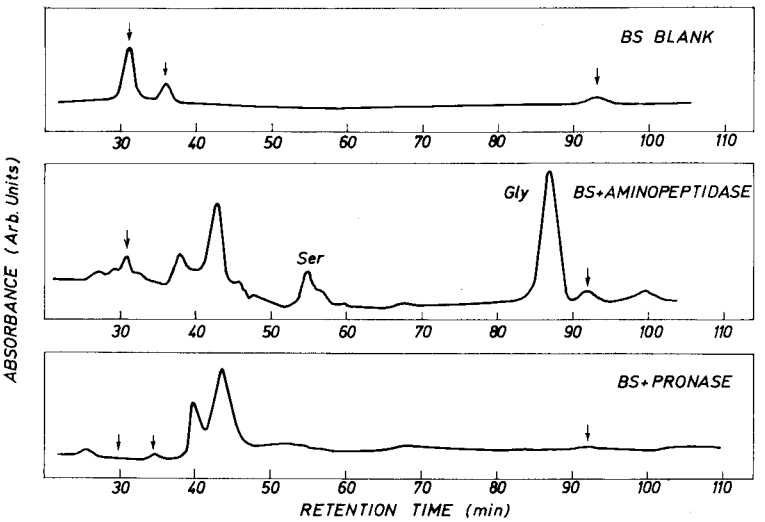


Fig. 1. Action of aminopeptidase M and pronase B grade on BS fraction. Chromotograms were obtained at pH 3,2 and on long column of amino acid analzer. Arrows indicate peaks which are partly or completely digested. Amounts applied are approximately equivalent. Peaks due to pronase-blank are omitted for clarity

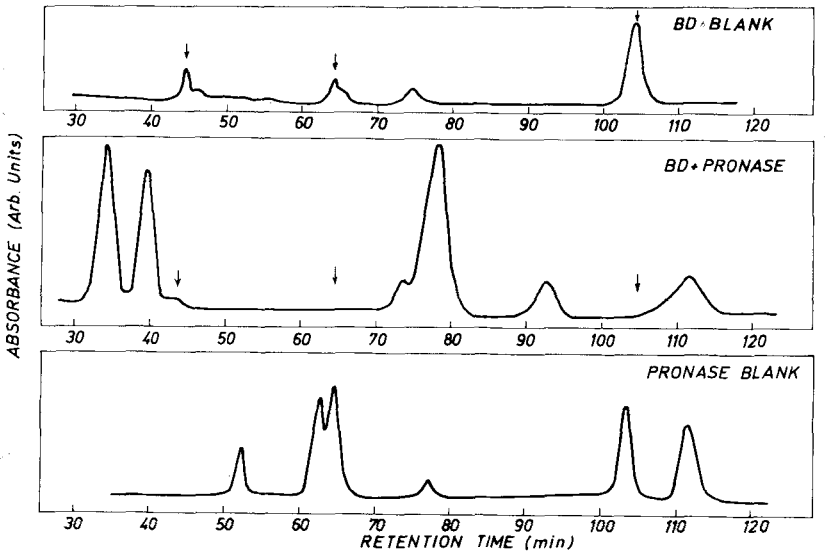


Fig. 2. Action of pronase B grade on BD fraction. Chromatograms were obtained on the amino acid analyzer's long column at pH 3.2, and after 90 min. at pH 4.2. Arrows indicate peaks which are digested partly or completely with pronase. Amounts applied are approximately equivalent

possible interaction between the enzyme and the radiolytic material. None of the peaks could be assigned to any of protein or nonprotein amino acids which were reported for this fraction in Table 2. This was also the case when the digested fraction was submitted to paper chromatography. A hexaglycine reference sample was only slightly digested under our working conditions. As one would expect from the lack of N-terminal amino acids, the aminopeptidase M did not react. HVE shows that fraction BD has a weak basic character.

Fraction AC. The acidic character of AC can be seen from Table 2: the acidic amino acids are more abundant (23%) than in any other fraction. Also, in HVE, several not completely resolved spots appear with mobilities between 0.4 and 2.2 relative to aspartic acid.

The appearance of bands at 1760 cm^{-1} and 1720 cm^{-1} in the IR spectrum (Table 3) accounts for the presence of dicarboxylic acids in the unhydrolyzed sample (Bellamy, 1975).

NE. This white powder appears on addition of ethanol to a concentrated solution of the neutral material (N) after DE-Cellulose. Its amount is small and increases on standing or further treatments such as heating or evaporation of the solution. On paper chromatography it remains at the origin and gives a positive reaction with both the Cl_2/I_2 and DBA reagents. Its amino acid content is similar to that of the original mixture (Table 2).

Urea. The identification was made by several methods (paper chromatography, amino acid analysis, and IR spectra). The digestion with urease was used for its quantitative elimination and determination. It has been found that urea represents a significant part (25.9%) of the total radiolytic mixture.

Fraction NUS1. This colorless, oily material constitutes 16.4% of the original mixture. A weak absorbance in the 220–230 nm region, trace amounts of glycine in the hydrolysate, and the absence of the Amide I band (1660 cm^{-1}) in the IR spectrum, clearly show the absence of peptidic properties.

In order to get a better insight into the chemical nature of the oligomer we have recorded the IR spectrum of both the deuterated and normal sample. Table 4 shows the results of an analysis of the recorded IR spectra.

A functional group analysis points to an abundant presence of the NH group. Bands at 3370 and 3180 cm^{-1}, which are shifted on deuteration to 2500 cm^{-1} and 2340 cm^{-1}, could be assigned to the stretching modes of hydrogen bonded NH groups and are characteristic of an imino group. This is also the case with bands at 1625 and 650 cm^{-1}, which originate from the in-plane and out-of-plane bendings of imino group. The presence of the CH_2 group is evident from the doublet at 1930 cm^{-1}, while the splitting of the deformation mode of the CH_2 and CH groups, in intervals of 1500–1300 cm^{-1} and 1100–900 cm^{-1}, may account for different chemical environments of such groups. It may be concluded that the oligomer giving the IR spectrum is an oligoimine, very likely a crosslinked one.

Table 4. Relative intensities of characteristic IR bands of fraction NUS 1, obtained by the usual procedure (I_ν/I_{2930}) and after deuteration $(I_\nu/I_{2930})_D$

$\nu_{max}(cm^{-1})$	(I_ν/I_{2930})	$(I_\nu/I_{2930})_D$	Tentative assignement
3370	10,4	8,4	$\nu_{a\,NH}$
3180	1,6	1,0	$\nu_{s\,NH}$
2930 d	1,0	1,0	ν_{CH}
2500	– –	1,2	$\nu_{a\,ND}$
2340	– –	0,1	$\nu_{s\,ND}$
1625	0,2	0,2	δ_{NH}
1450	0,1	0,1	δ_{CH}
1400 d	0,6	0,1	δ_{CH}
1315	0,5	0,2	
1225	0,3	0,2	ν_{CN}
1100	1,0	0,4	δ_{CH}
1036	1,4	0,8	δ_{CN}
980	0,2	0,2	δ_{CH}
650	– –	– –	δ_{NH}

The strong absorbance for biuret reaction in the fraction NUS1 is worthy of notice. The method used here (Ellman, 1962) is known to be characteristic of the peptide bond, but some amines also react: ethylenediamine, ethanolamine, and glucosamine. It is probable that some oligoimines in our sample also might react under conditions used in the routine procedure. This might explain also, why in some other fractions the estimates of amide are considerably higher than the values expected from amino acid analyses.

Fraction NUS2. The overlapping of NUS1 and NUS3 is strong and the material needs a further purification before a more detailed analysis. Data in Tables 1 and 3, however, lead to the conclusion that the fraction is not of particular interest, hence it has not so far been further examined.

Fractions NUS31 and NUS32. They were obtained from NUS3 by repeating the procedure on the Sephadex column. The fraction NUS31 is a white crystalline powder where the amino acid content represents 23% of the total weight of the fraction, in good agreement with prediction based on the biuret method. Amino acid analysis (Table 2) shows the presence of several protein and nonprotein amino acids besides some unidentified compounds.

Pronase did not act under the usual conditions.

Fraction NUS4. It is a white crystalline material which represents a considerable amount of the original mixture (18.4%) and is rich in amino acids (52% of the fraction weight). An estimate by biuret method (Table 1) suggests that most of the amino acids are not peptide bonded.

Preparative paper chromatography reveals the presence of seven constituents, NUS41–NUS47. The fractions NUS41 and NUS44–NUS47 are not of any particular interest to the present investigation and were not further considered.

Fraction NUS42. The assumption of a peptidic structure is supported by amino acid analysis data. One can calculate (Table 2) the minimal number of amino acid residues: 5 histidine, 1 A_{46}, 3 aspartic acids, 1 β-CH_3-aspartic, 1 serine, 3 sarcosines, and 4 glycines. From the peptide bond estimate, based on biuret data and using 800 M^{-1} cm^{-1} for molar absorptivity, one can calculate 1.3 peptide bonds per 3 amino acid residues, which is almost the theoretical value. These data suggest that the peptide fragment has a molecular weight of about 1600, while the oligomer could be as high as 5000.

Pronase did not act under the usual reaction conditions.

The infra-red spectrum (Table 3) has a pattern very similar to that of oligopeptides.

Fraction NUS43. This white crystalline substance represents 8.2% of the original mixture of products and is interesting in many respects. After hydrolysis glycine represents about 55% of its mass. The material completely lacks peptidic characteristics: it exhibits almost no absorbance in the 220–230 nm range, and the biuret reaction is negative.

Amide II/Amide I: Relative Intensity Ratios. IR spectra of neutral fractions NUS2, NUS31, NUS32 and NUS42 resemble the spectra of oligoamides in many details. They show absorption in the regions characteristic for peptide/amide bonds with variations in complexity and intensity of bands, particularly of those in the 1700–1500 cm^{-1} region.

A qualitative information on the structure of the oligomer is made possible by calculating the ratio of the Amide II/Amide I relative intensities. It is known that for polypeptides it equals 0.4–0.5 (Blout et al., 1961); it should be larger for oligoamides, where the extra NH group in the repeating unit contributes to a larger influence of Amide II absorption. We have found that this ratio varies from 0.73 to 2.2 for these fractions, where the total amino acids content in the hydrolysate varied from 30% to 9%, respectively. A reasonable guess is that the nonpeptidic part of the oligomer is an oligoamide, most probably an urea-aldehyde type. The release of ammonia during fractionation might also support this assumption.

Discussion

The procedures used in the present work for the preparation of the sample, and its irradiation and fractionation, have made the investigation of radiolytic products effective. At the same time, they have minimized the contribution of chemical changes that might occur without radiation. These changes, in alkaline ammonium cyanide, are known to be complex and their interpretations to some extent controversial (Ferris, 1979; Matthews, 1979).

The characterization of the fractionated materials has shown that most of the fractions are composite. Nevertheless, the present results are sufficient to get a reliable over-all picture of the chemical action of ionizing and of the chemical natures of the compounds formed. They show that the mixture of nonvolatile radiolytic products consists of urea (25.9%), of an oligomer (16.4%, NUS1) which is very likely an oligoimine, $-[NHCH_2]_n-$, and of several fractions (about 50%) that release amino acids on hydrolysis.

The search for constituents which have (or whose fragments have) peptidic properties was based on four tests: biuret reaction, IR spectra, amino acid assay, and enzymatic digestion. It has shown that the fractions releasing amino acids differ considerably:

– the fractions (BS, BD), where all four tests were found to be positive,

– the fractions (NUS31, NUS42), where no enzymatic cleavage was established, but where other findings strongly support the presence of peptidic fragments,

– a fraction (NUS43), very rich in glycine (55% of the fraction mass), which gives clearly negative results with biuret, IR spectra, and enzymatic digestion.

The analysis of the results also suggests that one of the important radiolytic products is an oligomer, very probably an urea-aldehyde oligomer, $-[NH\text{-}CO\text{-}NH\text{-}(CH_2)_x]_n-$. The peptidic skeleton, $-[NH\text{-}CHR\text{-}CO]_n-$, appears very likely as a fragment of urea-

Scheme 1. Formation of intermediates in irradiated aqueous ammonium cyanide

$$H_2O \longrightarrow OH, H, e^-_{aq}, H_3O^+, H_2, H_2O_2$$

$$HCN + OH \rightarrow HC(OH)=\dot{N} \text{ (or } H\dot{C}=NOH) \qquad k_1 \leqslant 5 \times 10^7\ M^{-1}\ sec^{-1} \qquad (1)$$

$$CN^- + OH \rightarrow \dot{C}(OH)=N^- + H_2O \rightarrow \dot{C}(OH)=NH + OH^- \qquad k_2 = 7 \times 10^9\ M^{-1}\ sec^{-1} \qquad (2)$$

$$C(OH)=NH \rightarrow NH_2\dot{C}=O \qquad k_3 = 4 \times 10^7\ M^{-1}\ sec^{-1} \qquad (3)$$

$$HCN + H \rightarrow HC(H)=\dot{N} \text{ (or } H\dot{C}=NH) \qquad k_4 = 3.6 \times 10^7\ M^{-1}\ sec^{-1} \qquad (4)$$

$$HCN + e^-_{aq} \rightarrow (HCN)^- \qquad k_5 = 6.6 \times 10^8\ M^{-1}\ sec^{-1} \qquad (5)$$

$$(HCN)^- + H_2O \rightarrow HC(H)=\dot{N} \text{ (or } H\dot{C}=NH) + OH^- \qquad k_6 \text{ very fast} \qquad (6)$$

$$NH_3 + OH \rightarrow \dot{N}H_2 + H_2O \qquad k_7 = 1 \times 10^8\ M^{-1}\ sec^{-1} \qquad (7)$$

aldehyde oligomers. The structure and molecular weight of the oligomers are unknown; the data on the fraction NUS42 suggest 1600 for the molecular weight of the peptide fragment and a MW up to five thousand for the oligomer.

Role of Free-Radicals in the Radiolysis of Aqueous Cyanide

The chemical action of ionizing radiation is based on the formation of short-lived reactive species, free-radicals, and radical-ions, which react with one another and with other solutes. These reactions lead to the production of reaction intermediates, which are observed by fast techniques during radiolysis, and to the formation of stable products that are identified by routine methods of chemical analysis after irradiation.

When a 0.1 M solution of ammonium cyanide is exposed to ionizing radiation the energy is deposited almost entirely in the water. Water molecules are excited and ionized and, as a result of a sequence of very fast reactions, hydroxyl radicals (OH), hydrogen atoms (H), and hydrated electrons (e^-_{aq}) are formed. The behavior of these chemically reactive species is well understood (Draganić and Draganić, 1971). Their reactions with hydrocyanic acid and cyanide ion consist of an attack on the triple carbon-nitrogen bond which leads to the formation of free-radical intermediates (Ogura, 1967; Ogura et al., 1972; Draganić et al., 1973; Behar, 1974; Draganić et al., 1976b; Büchler et al., 1976; Bielski and Allen, 1977). Scheme 1 summarizes these reactions. Ammonia reacts efficiently with OH radicals only (Pagsberg, 1972). Its reactions with H and e^-_{aq} species, like the corresponding ones of CN^-, are not included in the reaction scheme: they can be neglected since they are much slower than those given by Eqs. 4 and 5, where practically all the hydrogen atoms and hydrated electrons are consumed.

Scheme 2. Reactions of intermediates

$$2H_2C{=}\dot{N} \text{ (or } 2HC{=}\dot{N}H) \rightarrow HCN + H_2C{=}NH \ (+ H_2O \rightarrow HCHO + NH_3) \quad (8)$$

$$NH_2C{=}O + \dot{N}H_2 \rightarrow NH_2C(=O)NH_2 \quad (9)$$

$$HC(OH){=}\dot{N} \text{ (or } H\dot{C}{=}NOH) + HCN \text{ (or } CN^-) \begin{cases} \rightarrow HC(H){=}\dot{N} + HOCH \ (+ H_2O \rightarrow NH_3 + CO_2) & (10) \\ \rightarrow HC(H){=}\dot{N} + HNCO \ (+ NH_3 \rightarrow H_2NCONH_2) & (11) \end{cases}$$

$$H_2C{=}\dot{N} \text{ (or } H\dot{C}{=}NH) + n\ HCN \text{ (or } CN^-) \rightarrow \text{Oligomers} \quad (12)$$

$$HC(OH){=}\dot{N} \text{ (or } H\dot{C}{=}NOH) + n\ HCN \text{ (or } CN^-) \rightarrow \text{Oligomers} \quad (13)$$

The intermediates formed in reactions 1–7 are chemically reactive species which have under our working conditions half-lives on the sub-second time scale. Their absorption spectra were determined in pulsed-electron beam experiments by using setups for fast kinetic spectrophotometry. These experiments are characterized by high dose rates, where the concentrations of intermediates are relatively high, and the second order processes of their disappearance predominate. At lower dose rates, such as those in the gamma radiolysis, these reactions are in competition with the reactions of intermediates with hydrocyanic acid and cyanide ions. Scheme 2 presents the fates of intermediates which were formed in reactions 1–7, by taking into account the observations in fast kinetic spectrophotometry and the findings on stable radiolytic products in γ-radiolysis.

Urea is one of the most abundant compounds in irradiated cyanide. A probable pathway leading to its formation consists of a sequence of reactions, 2,3, 7 and 9. HNCO is unstable and the reaction 11 is less likely, but cannot be ruled out.

The importance of reactions 12 and 13 for the radiolytic formation of carbon-nitrogen compounds is suggested by the previous finding that about 70% of nitrogen from decomposed cyanide is in the mixture of nonvolatile products (Draganić et al., 1977a). The results of the present analyses show that at least three types of oligomers are formed: oligoimine, urea-aldehyde, and an oligomer with a peptide linkage. The correlation of various chemical changes with absorbed dose that was shown in our previous work (intensity of some IR bands, concentration of amino acids, intensity of biuret reaction), suggests that oligomerizations already start at small radiation doses and do not seem to be significantly disturbed by the accumulation of radiolytic products that take place at larger doses. The structures of oligomers produced by reactions 12 and 13 are unknown. The absence of IR bands characteristic of CN groups rules out the presence of the oligomer

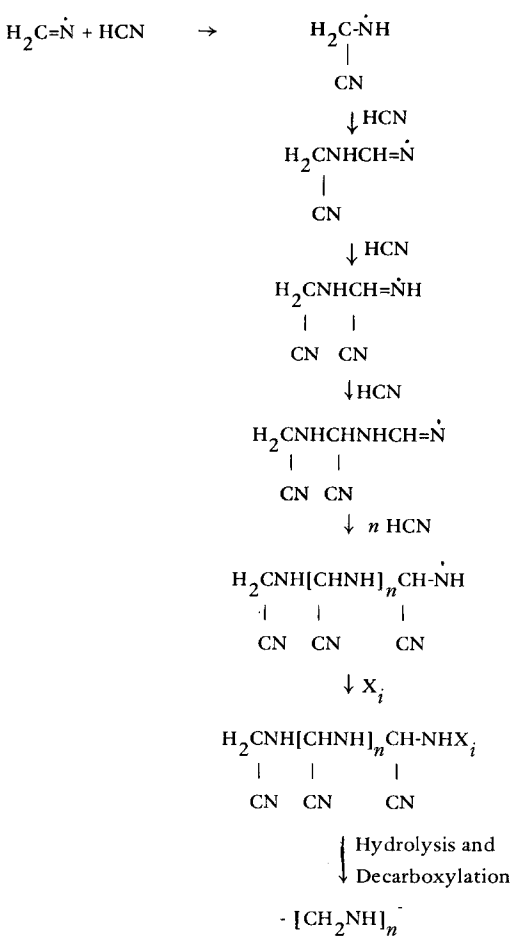
$$-\left[\begin{array}{c} NH_2 \\ | \\ C \\ | \\ CN \end{array}\right]-$$

which was considered previously (Völker, 1960; Draganić et al., 1976b; 1977a).

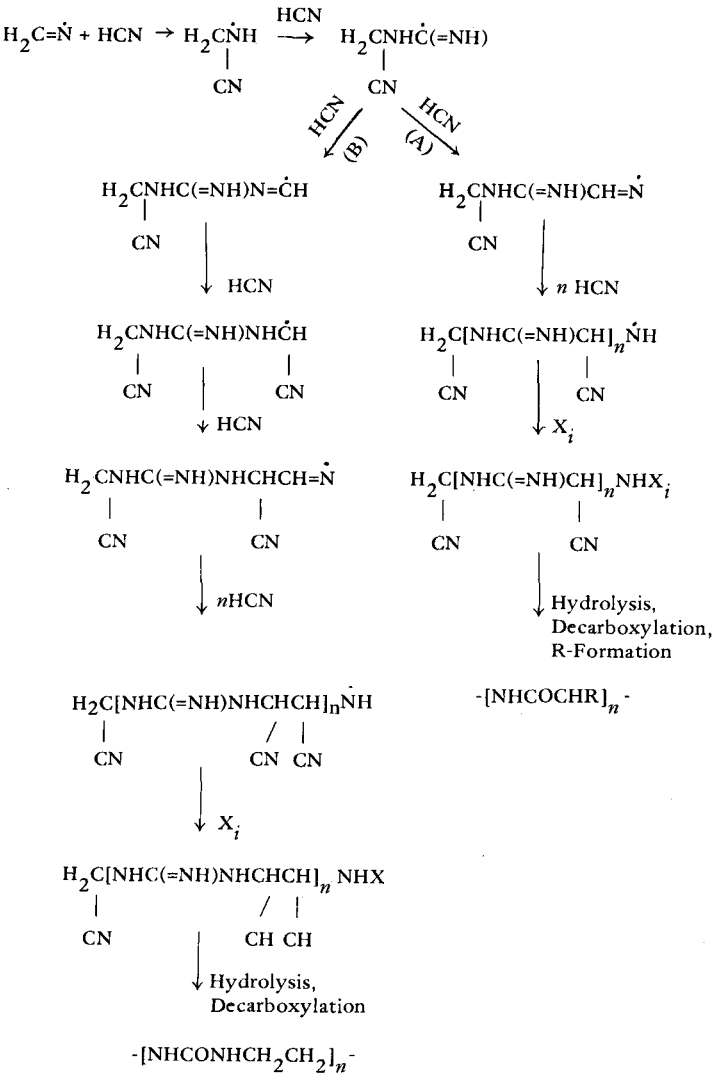
Various findings in this work enable one to consider probable reaction schemes leading to the oligomers formed in irradiated samples. In principle, we have here as the predominant process an additional oligomerization. It consists of the addition of one reactive species to another via utilization of unsaturated valences and free-radicals. The free radicals initiate a short chain reaction in the irradiated aqueous cyanide; the additional step consists of the addition of hydrogen cyanide, which might take place in several ways, and the chain is terminated by a radical-radical reaction.

Scheme 3 represents a possible pathway leading to the formation of oligoimine in the fraction NUS1. The oligomerization is initiated by free-radicals with a spin-unpaired electron on the nitrogen atom, $H_2C{=}\dot{N}$ and $HC(OH){=}\dot{N}$. Hydrogen cyanide adds in such a way that a free radical is always formed which has an unpaired electron

Scheme 3. Some hypothetical oligomerization reactions of formation of oligoimine



Scheme 4. Some hypothetical reactions of formation of urea – aldehyde oligomer with peptidic fragment



on the nitrogen. Its reaction with some of intermediates (X_i) from reaction 1–7 terminates the oligomerization process. The oligoimine appears after cyano groups have undergone hydrolysis and decarboxylation.

The present results suggest that constituents releasing amino acids represent a relatively small part of isolated fractions, at best 30%, and that they are very likely fragments, most probably side chains, of urea-aldehyde oligomers. Possible pathways leading to these radiolytic products are presented in Scheme 4. As distinct from the case of oligoimine considered above, the oligomerization can be initiated here by intermediates with a spin unpaired electron both on nitrogen and on carbon atoms. The

addition of hydrogen cyanide takes place in two different ways (A and B), and additional steps lead to the formation of larger radicals with an unpaired electron on alternatively N and C atoms. Their reactions with some of intermediates (X_i) produced by reactions 1–7 terminate the oligomerization process. The hydrolysis of cyano groups, followed by decarboxylations, gives the urea-aldehyde oligomer (B) with a peptidic fragment (A). The complex side chain (R) from amino acids might originate from the activated cyano group. Additions through the pathway B occur more easily than those leading to a peptidic structure through A. Also, the activated cyano group may be the place where the branching of oligomer A starts.

Radiolytic Products Releasing Amino Acids

A 75% decomposition of ammonium cyanide, such as in most of the present experiments (18 Mrad dose), gives about 2.8 grams of radiolytic products per 1 liter of sample (0.1M, pH 9). About 15% of this material appears on hydrolysis in several protein and nonprotein amino acids.

Glycine was the most abundant amino acid in all fractions; its contribution to their total amino acid contents was found to vary from 21% (NUS42) to 100% (NUS43, NUS44). At least two different pathways are leading to the formation of glycine in the radiolysis of aqueous ammonium cyanide. One, unknown, concerns glycine in the fraction NUS43, where it constitutes as much as 55% of the hydrolysate and is not peptide bonded. Another is in the oligomerization process which is initiated by free-radical intermediates and followed by hydrolyses of cyano groups and subsequent decarboxylations. This was the case with the fractions BS and BD, and might be also the case with the fractions NUS31 and NUS42.

Present experiments show that we were not able to isolate pure oligopeptidic material with physico-chemical methods used for fractionations in this work. Results of analyses suggest that the peptide-bonded material appears as a compact fragment of the urea-aldehyde oligomer. This is supported by IR spectra, which also give 10^3 as a rough estimate for the fragment's molecular weight. The amino acids in peptidic fragment are α-peptide-bonded. The supporting evidence is given by the biuret reaction and in some cases (fractions BS and BD) by the enzymatic cleavage.

It is known that the formation of peptide bonds by condensation of amino acids in aqueous medium is a thermodynamically unfavorable process, since the removal of H_2O during condensation has to take place in the presence of a large amount of water. The findings in the present work, concerning oligomers with fragments containing a peptidic skeleton, are of interest to studies of chemical evolution, since oligomers are formed in aqueous medium and without the intervening formation of amino acids. Further work concerns the purification of isolated materials and the examination by enzymatic hydrolysis of their behavior.

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