Carbon-13 NMR Study on the Combination of Formaldehyde with Bovine Serum Albumin

Daniel Tome,* Andre Kozlowski, and Francoise Mabon

^{13}C-Enriched formaldehyde bonds with bovine serum albumin (BSA), reductively methylated BSA, and S-carboxymethylated BSA were observed directly by using carbon-13 NMR spectroscopy. Pronase digestion of the treated proteins was useful to improve the resolution of the intensified signals. Evidence is presented of four mechanistic pathways implicating lysine through its *N*-hydroxymethyl derivative. The major reaction is the formation of a lysine–arginine cross-link. The amide groups of asparagine or glutamine also react with lysine and formaldehyde with the formation of lysine–amide cross-link and (hydroxymethyl)amide adducts. A Mannich-like compound linking, through a methylene bridge, lysine and tyrosine side chains has been observed. In addition, methyllysine and formyllysine are formed. The last three bonds are acid resistant; the others are acid labile. Tryptophane, histidine, and tyrosine derivatives are also postulated, but their structure is not yet established.

INTRODUCTION

Formaldehyde is a protein cross-linking reagent extensively used in tanning industries (Gustavson, 1956; Walker, 1964). In medical science, it is also used for the preparation of toxoids, which are injected into humans; it is therefore imperative to provoke irreversible modifications, masking the toxicity but preserving the antigenic site (Bizzini and Raynaud, 1974). More recently, it has been used in animal feed technology for the rumen bypass of dietary proteins (Ferguson et al., 1967; Zelter et al., 1970) or to encapsulate lipids into protein shells (Scott et al., 1971; Wrenn et al., 1975); in those cases, it is necessary to form bonds which reduce protein solubility and deamination in the rumen but are reversible in the lower part of the digestive tract, without liberation of undesirable compounds stored in edible animal products. On the other hand, formaldehyde is suspected to be involved in the hypermethylation that affects proteins in biological systems during malignant cell proliferation (Tyihak et al., 1980) and in the toxic effect of alcohols by an inactivation of several alcohol deshydrogenases (Grisolia et al., 1975). Because of these implications in medical science and in animal feeding, it is important to know exactly the various compounds that may be formed during the reaction of formaldehyde with proteins, their conditions of formation, and their degree of reversibility. Now these aspects are not yet entirely understood.

According to studies with model compounds, the initial reaction leads to the fast formation of hydroxymethyl derivatives with reactive groups on amino acid side chains. This addition is particularly important with lysine but also occurs with cysteine, arginine, asparagine, glutamine, serine, histidine, tryptophane, and tyrosine (French and Edsall, 1945; Dunlop et al., 1973; Kitamoto and Maeda, 1980; Tome and Naulet, 1981; Tome et al., 1982; Naulet and Tome, 1983). Moreover spontaneous methylation and formylation proceed on the side chain of lysine (Tyihak et al., 1980; Tome et al., 1982; Trezl et al., 1983). Some hydroxymethyls may evolve to methylene bridges; they all implicate lysine or cysteine which are postulated to be linked either together or with arginine, aspargine, or glutamine; in addition, lysine reacts with the phenol ring of tyrosine, indole ring of tryptophane, and imidazole ring of histidine (Fraenkel-Conrat and Olcott, 1948a,b; Shao, 1970; Bizzini and Raynaud, 1974; Dewar et al., 1978; Koga et al., 1978; Taylor et al., 1978; Naulet and Tome, 1984; Tome et al., 1984). These methylene bridges are introducing intra- and intermolecular cross-linkages in proteins, leading to their polymerization, a decrease of their solubility and degradability by proteolytic enzymes, together with a modification of various others properties (Davis and Tabor, 1963; Hopwood, 1969; Galembeck et al., 1977; Tome et al., 1979).

The analysis of formaldehyde-treated proteins shows that after reaction formaldehyde is bound at different levels called "reversible", "acid labile", and "acid resistant" (Bizzini and Raynaud, 1974; Tome et al., 1979). The identification of the corresponding derivatives has generally been made by chromatographic methods after acid or enzymic hydrolysis of formaldehyde or [^{14}C]-formaldehyde treated proteins. Various derivatives have been proposed including methyllysine (Reis and Tunks, 1973; Tome et al., 1979; Nakai et al., 1980), (hydroxymethyl)-glutamine (Caldwell and Millighan, 1972), a cross-link between lysine and tyrosine (Bizzini and Raynaud, 1974), between two lysine (Caldwell and Millighan, 1972), or between serine and asparagine, or glutamine (Meyers and Hardman, 1971). However, the methods used in order to identify these products do not provide unequivocal proof of their formation because of important modifications that may interfere during hydrolysis; this difficulty is amplified by the reversibility of a large part of the links and their very low concentration in the presence of a polypeptide chain. These difficulties are overcome by using ^{13}C-enriched formaldehyde and ^{13}C NMR spectroscopy (Taylor et al., 1978; Jentoft et al., 1979). This technique has been used for a direct observation of the reaction products of formaldehyde with proteins and was useful, from the chemical shift analysis, to hypothesize the formation of (hydroxymethyl)lysine, (hydroxymethyl)histidine, (hydroxymethyl)asparagine or glutamine, methyllysine, and a lysine–arginine methylene bridge as major products (Tome et al., 1984).

In the present paper, bovine serum albumin (BSA), used as a protein model, has been treated by ^{13}C-enriched formaldehyde before and after specific modification of lysine and cysteine residues, in order to identify by NMR spectroscopy the sites that participate in the formaldehyde–BSA combination and the nature and stability

National Institute for Agricultural Research, Laboratory of Animal Feed Technology, 44072 Nantes, Cedex, France (D. T. and A. K.), and University of Nantes, Organic Chemistry, E.R.A. C.N.R.S. 315, 44072 Nantes, Cedex, France (F. M.).

0021-8561/85/1433-0449$01.50/0

of the corresponding bonds before and after enzymic or acid hydrolysis.

EXPERIMENTAL PROCEDURE

Materials. N^{α}-acetyl amino acids (protected amino acids), poly-L-lysine, and pronase E (70.000 PUK/g) (Sigma) were used without additional purification. Bovine serum albumin (BSA) (Sigma) has been extracted with hexane. A 10 M solution of formaldehyde has been prepared by depolymerization of pure paraformaldehyde (Merck) in distilled water at 85 °C. A 6.5 M solution of [^{13}C]-formaldehyde (91 % enriched), stabilized by methanol, was obtained from Prochem. The synthetic Mannich-like compound N^{ϵ}-lysyl-C(3)-tyrosylmethane, linking through a methylene bridge lysine and tyrosine side chains, was prepared from protected lysine and tyrosine as previously described (Tome et al., 1984). Reductively methylated BSA (BSAm) was prepared according to Means and Feeney (1968) and S-carboxymethylated BSA (BSAc) according to Crestfield et al. (1963).

Sample Preparation. Model mixtures were prepared by allowing poly-L-lysine (30 g/L) and a protected amino acid (0.5 M) to react with [^{13}C]-formaldehyde (0.5 M) in water at 37 °C and pH 7 (adjusted with HCl or NaOH) for five days.

Proteins were dissolved in phosphate buffer (0.1 M, pH 7), in water, or in deuterated water at a concentration of 100 g/L (BSA and BSAm), or 10 g/L (BSAc) in the presence of formaldehyde or [^{13}C]-formaldehyde (3.3 mmol/g of protein). Solutions were incubated for five days at 37 °C and dialyzed against water or deuterated water to eliminate "free" and "reversibly bound" formaldehyde.

Enzymic hydrolysis was performed by adding pronase E (15 mg/g of protein) to the dialyzed solutions of proteins and incubation for 24 h at 37 °C. The hydrolysates were then freeze-dried.

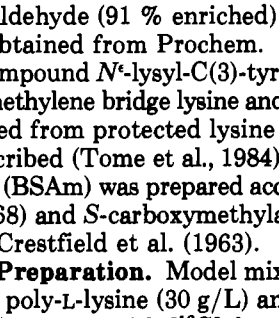
"Acid-labile" formaldehyde from treated BSA was eliminated, after dialysis, by steam distillation in the presence of phosphoric acid (Tome et al., 1979). The phosphoric acid was then eliminated on a column (90 × 2.5 cm) of Sephadex G25 eluted with water and the excluded fraction (proteins) was freeze-dried.

Acid hydrolysis of treated BSA was performed after elimination of "acid-labile" formaldehyde in 6 M hydrochloric acid for 24 h at 110 °C. The hydrolysate was concentrated under reduced pressure at 40 °C and redissolved in water (100 g/L).

Hydrochloric hydrolysate was fractioned by ion exchange chromatography on a column (30 × 2.5 cm) of Dowex 50X4 equilibrated with volatile pyridine acetate buffer (0.3 M, pH 4.7) and regenerated with pyridine acetate buffer (2.23 M, pH 5.3). Absorbance was measured on an aliquot of each fraction at 560 nm after reaction with ninhydrine. Isolated fractions were concentrated under reduced pressure in order to eliminate pyridine acetate and redissolved in water.

Analytical Techniques. Carbon-13 NMR spectra were recorded in the pulsed Fourier transform mode on a Bruker WM 250 spectrometer operating at 62.896 MHz and equipped with a 10-mm probe. Data were gathered in quadrature-phase mode by using the 16 K frequency domain data point (spectral width 12 000 Hz; acquisition time 0.5407 s; delay time 0.10 s; flip angle 30°). A broad band heteronuclear noise decoupler was used to give proton decoupled spectra. The use of resonance decoupling and of the APT pulses sequence (Attached Proton Test) was of help for the attribution of resonances. Samples (3.0 mL) were placed into a standard 10-mm NMR sample tube. For samples in deuterated water, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference. When the solvent was water, deuterated benzene (C_6D_6) put into a coaxial tube was used as external reference and the field frequency lock was provided by C_6D_6. Carbon-13 chemical shifts were corrected to values relative to tetramethylsilane (Me_4Si) by using −2.1 ppm as the position of the DSS resonance relative to Me_4Si and 128 ppm as the position of C_6D_6. In general, reported peaks have a limiting accuracy of 0.05 ppm. The probe temperature was 30 ± 1 °C.

Table I. ^{13}C Chemical Shifts (Me_4Si) of the Carbon Coming from [^{13}C]-Formaldehyde in Model Compounds Prepared by Reacting Poly-L-lysine (RNH) with [^{13}C]-Formaldehyde Alone (1) or in the Presence of Protected Arginine (2), Glutamine (3), Cysteine (4), or Tyrosine (5)[a]

	nature of the carbon	chemical shift, ppm	designation
1	RNHCHO	164.5	J
	$RNHCH_2OH$	71.7	
	$RN(CH_3)_2$	43.2	
	$RNHCH_3$	33.1	A
2		65.4	
		64.8-64.2	K
		66.3 (a)-59.1 (b)	G-Eg
		59.8	E
3	Gln-$CONHCH_2OH$	63.2	F
	$RNHCH_2NHCO$-Gln	51.8	D
4	Cys-SCH_2OH	65.7	
	$RNHCH_2S$-Cys	52.7	
	$RN(CH_2S\text{-Cys})_2$	57.9	
5	$RNHCH_2$-Tyr	47.0	C
	$RN(CH_2\text{-Tyr})_2$	56.8	L

[a] Proposed structures.

Amino acids were analyzed by using standard conditions with a Kontron Liquimat III autoanalyzer equipped with a column (30 × 0.4 cm) of cationic resin Durum DC 6A. Basic amino acids and specific derivatives were separated with a sodium citrate buffer (0.35 M, pH 4.7) at 34.5 °C at a flow rate of 18 mL/h and identified by comparison with a standard solution containing tyrosine, phenylalanine, lysine, monomethyllysine, dimethyllysine, histidine, 1-methylhistidine, 3-methylhistidine, N^{ϵ}-lysyl-C-(3)-tyrosylmethane and arginine merging from the column in that order.

RESULTS AND INTERPRETATION

Bonds between formaldehyde and BSA were studied after dialysis of the treated protein, after pronase digestion, and after elimination of acid-labile bound formaldehyde. In each case, ^{13}C NMR spectra of formaldehyde and [^{13}C]-formaldehyde treated protein were recorded and chemical shifts of the intensified signals compared to values obtained from model compounds (Table I) prepared by known reactions previously described (Tome and Naulet, 1981; Tome et al., 1982; Naulet and Tome, 1984; Tome et al., 1984). In addition, the nature of these intensified signals was determined, when possible, by the use of the APT pulses sequence.

In a preliminary study, it was observed by NMR that some intensified signals were eliminated from spectra after dialysis of the [^{13}C]-formaldehyde treated BSA; these

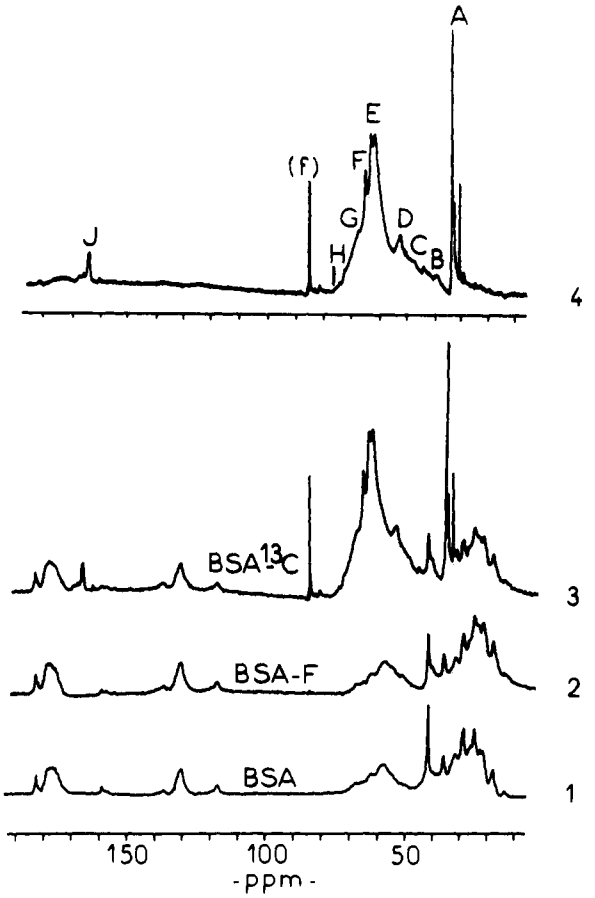


Figure 1. The carbon-13 NMR spectra of BSA, formaldehyde-treated BSA (BSA-F) and [^{13}C]-formaldehyde-treated BSA (BSA-^{13}C). Spectrum 1: BSA at 100 g/L in phosphate buffer (0.1 M, pH 7, 80 000 scans). Spectra 2 and 3: BSAF-F (2) or BSA-^{13}C (3) at 100 g/L after 5 days incubation at 37 °C and dialyzed (80 000 scans). Spectrum 4: obtained by digitally substracting spectrum 2 from spectrum 3; f, residual formaldehyde; A, B, C, D, E, F, G, H, I, J, intensified signals.

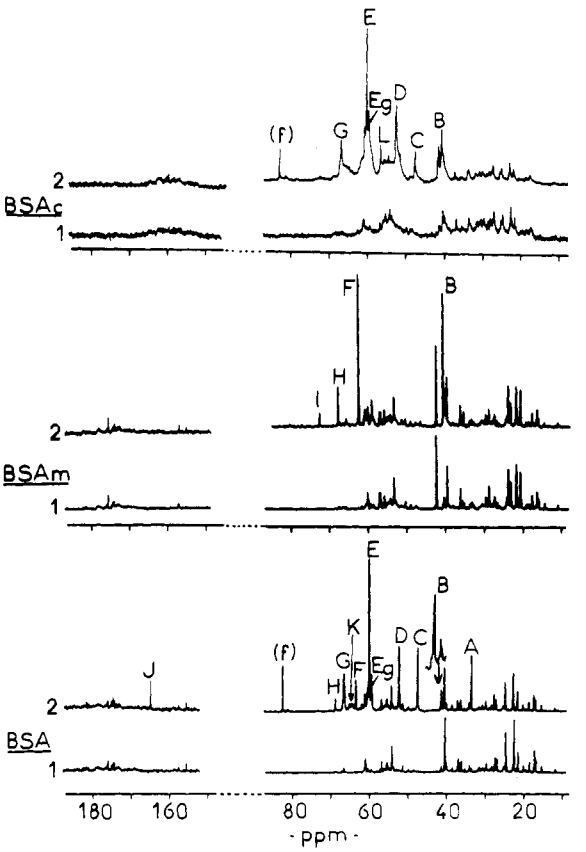


Figure 2. The carbon-13 NMR spectra obtained from solutions (100 g/L) of BSA, reductively methylated BSA (BSAm), or S-carboxymethylated BSA (BSAc) after treatment with formaldehyde (1) or [^{13}C]-formaldehyde (2), 5 days at 37 °C, dialysis, and hydrolysis by pronase. Each spectrum results from 80 000 scans: f, residual formaldehyde; A, B, C, D, E, Eg, F, G, H, J, K, L, intensified signals.

signals were assigned to formaldehyde (82.3 ppm) and methanol (49.3 ppm) of the formaldehyde solution, formic acid (172.0 ppm), (hydroxymethyl)lysine (71.8 ppm), and N-terminal hydroxymethyl (70.2 ppm). The other peaks are more stable and have been studied in more detail.

NMR Spectra of Dialyzed Formaldehyde-Treated BSA. Typical NMR spectra obtained after dialysis of BSA, untreated and treated with formaldehyde (BSA-F) or ^{13}C-enriched formaldehyde (BSA-13C), are shown in Figure 1. Some differences between BSA and BSA-F appear in the range 20–30 ppm but are difficult to interprete: these two spectra are characteristic of an undenatured state of the protein. With BSA-^{13}C, various intensified peaks are observed and are better individualized on the spectrum resulting from the difference between BSA-^{13}C and BSA-F spectra. The intensified peaks are classified in ten groups identified as methyl (A), methylene (B, C, D, E), hydroxymethyl (F, G, H, I), and formyl (J) carbons. The signals are however not all resolved because of an important line broadening, due to the undenatured state of protein.

NMR Spectra of Formaldehyde-Treated BSA, BSAm, and BSAc after Pronase Hydrolysis. Digestion of treated proteins by pronase is a mild procedure to destroy the protein structure without cleavage of the formaldehyde–protein acid-labile bonds. In this way, the resolution of the intensified peaks is largely improved, all signals from the same chemical species, but in a different environment in the native protein, should merge to a single NMR signal. This method is useful to evaluate the influence of specific modifications of lysine (BSAm) or cysteine (BSAc) residues on the combination of formaldehyde with BSA (Figure 2).

Intensified methyl carbons merge in one signal A (33.1 ppm) after pronase digestion and formyl in one signal J (164.8 ppm). These two signals, A and J, which are not formed with BSAm, are respectively assigned to methyllysine and formyllysine; their absence with BSAc is rather explained by a mobilization of formaldehyde by other reactions, preventing their formation, and by the low concentration of reagents used in that case.

Among methylene carbons, peaks B merge in three signals (39.7, 40.9, 41.0 ppm) after pronase digestion; these signals are present either with BSAm or BSAc and, as a matter of fact, do not implicate either lysine nor cysteine. Considering these chemical shifts, it may be assumed that the compounds are methylene bridges, either between two amide groups, estimated to appear in the range 41–45 ppm (Kelly et al., 1977; Taylor et al., 1978), between an amide group and tyrosine, predicted in the range 42–45 ppm (Dewar et al., 1978), or, more probably, between an amino group and histidine or tryptophane, predicted in the range 38–42 ppm (Tome and Naulet, 1981; Kelly et al., 1977). These signals are unfavored with BSA.

The other methylene carbons, C, D, and E, which are present with BSAc and not with BSAm, do not implicate cysteine but all implicate lysine. Peak C (47.0 ppm), assigned to a lysine–tyrosine methylene bridge, clearly appears after pronase digestion. Peak D is resolved after enzymic hydrolysis in two signals (51.7, 51.8 ppm) assigned

Table II. Amino Acid Autoanalyzer and Carbon-13 NMR Analysis of the Fractions Separated on Dowex 50X4 from [^{13}C]-Formaldehyde-Treated BSA Hydrochloric Acid Hydrolysate

fraction	autoanalyzer amino acids analysis	carbon-13 NMR analysis: intensified signal, ppm	carbon-13 NMR analysis: nonintensified signals, ppm	carbon-13 NMR analysis: identified compound
1	acid and neutral amino acids, tyrosine, phenylalanine		complex spectrum	
2	lysine		174.8, 54.9, 39.4, 30.2, 26.6, 21.6	lysine
	N^{ϵ}-methyllysine	33.1 (A)	174.8, 54.9, 48.9, 30.2, 25.2, 21.6	N^{ϵ}-methyllysine
3	histidine		173.6, 134.8, 126.6, 118.8, 54.5, 25.4	histidine
	ammonia			
	?	30.8 (M)	?	?
4	N^{ϵ}-lysyl-C(3)-tyrosylmethane	46.9 (C)	174.7, 174.0, 154.9, 132.3, 127.3, 118.3, 116.2, 56.2, 54.8, 46.8, 35.5, 30.1, 25.2, 21.6	N^{ϵ}-lysyl-C(3)-tyrosylmethane
5	arginine		174.2, 157.1, 54.6, 41.0, 27.9, 24.3	arginine
6			complex spectrum	

to lysine–asparagine and lysine–glutamine methylene bridges. The important signal E (59.8 ppm) is assigned to a lysine–arginine methylene bridge which appears as the major product of the combination of formaldehyde with BSA; the presence of another species Eg (59.2 ppm), attributed to an hydroxymethylation on the free nitrogen of the guanidyl group, with the hydroxymethyl signal G (66.3 ppm), agrees with observations on model compounds. In addition the two small signals K (64.2, 64.8 ppm) that appear after pronase hydrolysis may be assigned to the lysine–arginine dimethylene ether also suspected to be formed in model mixtures. The other hydroxymethyls F (63.2 ppm), H (68.6 ppm), and I (72.1 ppm) are respectively assigned to amide (aspargine or glutamine), tryptophane, and histidine hydroxymethyls (Tome et al., 1981). Last, an additional peak L (55.8 ppm) was formed with BSAc and could be assigned to the link of two tyrosine molecules on lysine nitrogen.

Analysis of Acid-Resistant Formaldehyde–BSA Bonds. NMR spectra obtained after elimination by steam distillation of acid-labile formaldehyde from treated BSA and after hydrochloric acid hydrolysis are reported on Figure 3. After elimination of acid-labile formaldehyde, the only intensified signals that still remain on the spectrum were A, C, and J respectively assigned to methyllysine, a lysine–tyrosine cross-link, and formyllysine. When samples are subjected to hydrochloric acid hydrolysis, peak J disappears and a new methylene signal M (30.8 ppm) is observed.

The hydrochloric acid hydrolysate was chromatographied on a column of Dowex 50 × 4 (Figure 4). In the presence of ninhydrin, the elution profile reveals six fractions which were separately collected, analyzed on the amino acid autoanalyzer, and studied by NMR (Table II). The two intensified NMR signals A and C respectively appear in fractions 2 and 4 and the results clearly show the presence of methyllysine associated with signal A and of a lysine–tyrosine cross-link associated with signal C. On the other hand, it has been impossible to identify the origin of signal M, present in fraction 3, in which histidine and ammoniac were the only compounds detected. The corresponding derivative may be at too low a concentration and does not appear in the analysis. From its chemical shift, however, it could be assigned to a methylene bridge linking two tyrosines by the C(3) of the phenol ring; this methylene type is predicted near 30 ppm (De Breet et al., 1977).

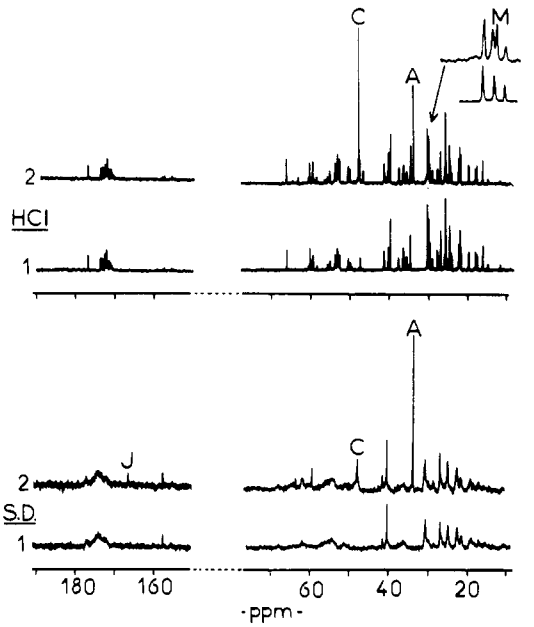


Figure 3. The carbon-13 NMR spectra obtained from solutions (100 g/L) of BSA after treatment with formaldehyde (1) or [^{13}C]-formaldehyde (2), 5 days at 37 °C, dialysis and steam distillation (SD), or dialysis, steam distillation, and 6 M hydrochloric acid hydrolysis (HCl). Each spectrum results from 80 000 scans: A, C, J, M, intensified signals.

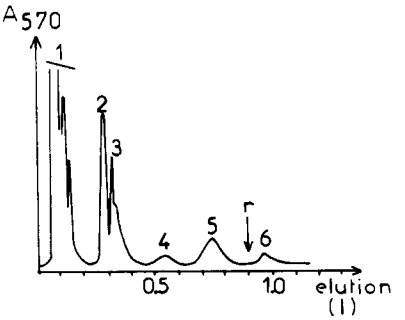
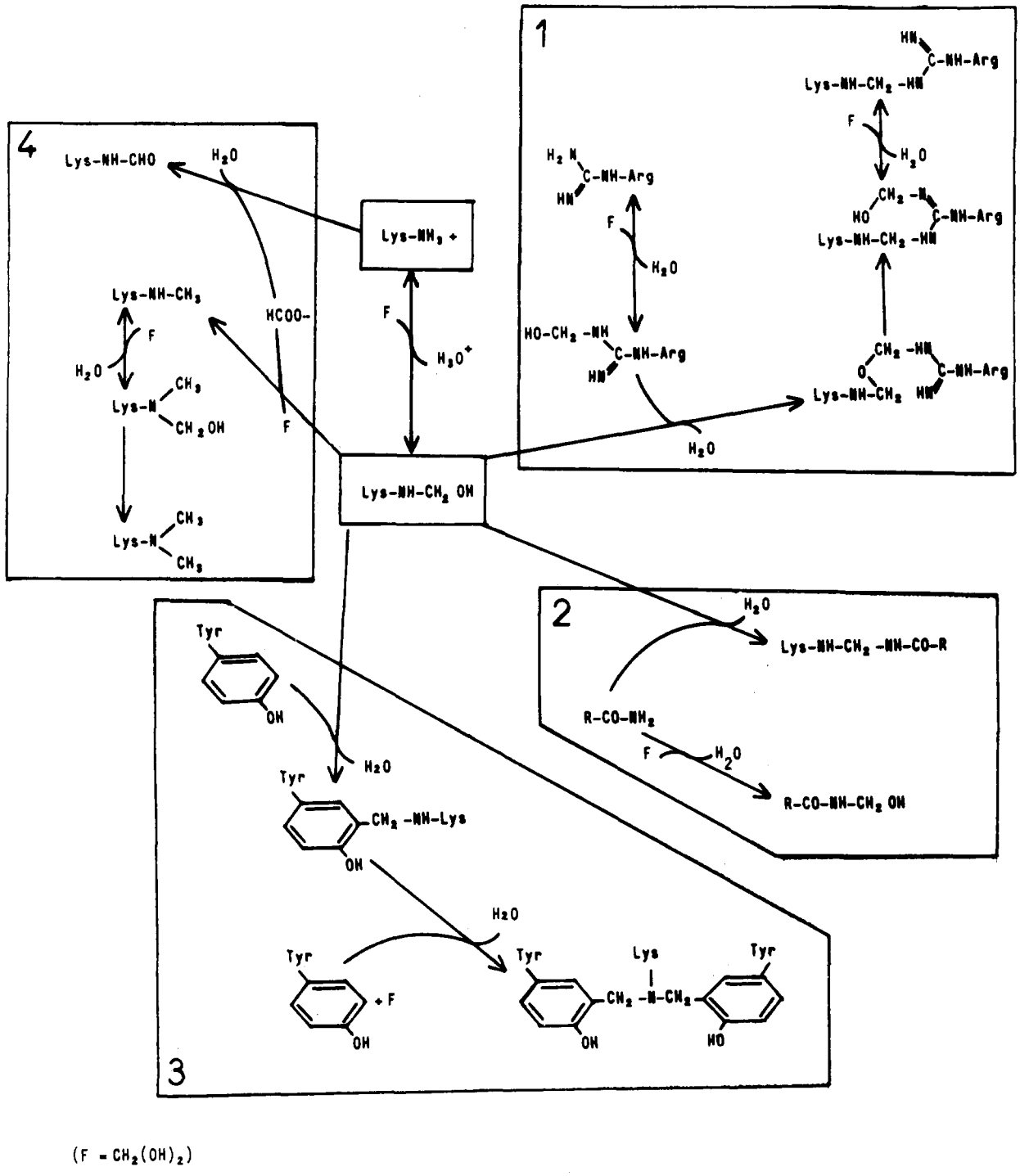


Figure 4. Elution profile from Dowex 50X4 ion exchange chromatography of a 6 M hydrochloric acid hydrolysate of [^{13}C]-formaldehyde-treated BSA: r, column regeneration.

DISCUSSION

Reaction Sites and Lability. Under the mild conditions used in this study and in agreement with previous hypotheses (Bizzini and Raynaud, 1974), formaldehyde is bound to BSA at various levels including reversible, acid labile, and acid resistant bonds.

The reversibly bound fraction is in the form of reversible *N*-(hydroxymethyl) adducts. This N-hydroxymethylation is particularly important on the ϵ-amino group of lysine but is also effective on the α-amino group of NH_2-terminal amino acids (Kitamoto and Maeda, 1980; Tome et al., 1982), the guanidyl group of arginine, and the ring nitrogen of histidine and tryptophane (Dunlop et al., 1973; Tome



(F = $CH_2(OH)_2$)

Figure 5. Proposed mechanistic scheme for the major formaldehyde–BSA reactions.

et al., 1981). These reversible reactions represent the main initial steps of the combination of formaldehyde with proteins under mild conditions (Means and Feeney, 1968; Feeney et al., 1975). The products quickly dissociate when formaldehyde is removed by dialysis.

The acid-labile formaldehyde is bound to BSA as hydroxymethyl adducts or methylene bridges. The major fraction is made of methylene bridges linking lysine to arrginine, asparagine, or glutamine; these species often thought to be formed (Fraenkel-Conrat and Olcott, 1948a; Taylor et al., 1978; Koga et al., 1978) were difficult to observe because of their lability. Acid-labile hydroxymethyls are mainly formed with the amide group of asparagine or glutamine and their identification confirms hypotheses previously put forth (Fraenkel-Conrat and Olcott, 1945; Caldwell and Millighan, 1972; Tome et al., 1981). The reversibly bound hydroxymethyl group on tryptophane is also able to be converted into a more stable hydroxymethyl by migration from the 1- to the 2-position of the imidazole ring (Fraenkel-Conrat et al., 1947; Shao, 1970). Last, additional methylene groups that do not implicate lysine have been detected but were not clearly identified; among several hypotheses, the most likely is the formation of methylene bridges linking the indole ring of histidine or the imidazole ring of tryptophane to other groups, possibly a N-terminal amino group, a peptidic linkage, or an amide group (Martin and Marini, 1967; Tome and Naulet, 1981). On the other hand, the amide–amide methylene bridge, already supposed to be formed (French and Edsall, 1945; Kelly et al., 1977; Taylor et al., 1978), was not observed on model mixture (Koga et al., 1978).

The acid-resistant fraction is made of methyllysine, formyllysine, and a lysine–tyrosine methylene bridge. Formyllysine is destroyed during the 6 M hydrochloric acid hydrolysis; the two other adducts remain in the hydrolysate and their presence is presently definitely established by their NMR analysis. These results confirm that formaldehyde is able to play a role in various mechanisms of lysine methylation and formylation in proteins (Reis and Tunks, 1973; Tome et al., 1979; Tyihak et al., 1980; Trezel et al., 1983). It has also been proved that the lysine–tyrosine methylene bridge represents the irreversible step in the formaldehyde–protein cross-linking as already proposed (Bizzini and Raynaud, 1974). Another tyrosine–tyrosine methylene bridge could be formed during acidic hydrolysis.

Mechanistic Discussion. From the above results and according to current studies, the amino group on the lysine side chain is largely involved in the formaldehyde–BSA combination. Although not yet complete, four basic mechanistic pathways acting on this group under mild conditions may be proposed (Figure 5).

The major pathway is the lysine–arginine methylene bridge formation. A presumed mechanism is first a condensation step between two hydroxymethyls leading to a methylene ether cross-link. In a second step, elimination of formaldehyde through a six-membered transition state gives a methylene cross-link. This hypothesis is supported by the assumed presence of the various intermediates as well in model mixtures (Tome et al., 1984) as with proteins. It is also consistent with previous results suggesting that, for the major formaldehyde–protein product, two molecules of formaldehyde are implied in the transition state, only one in the product, water being eliminated during the reaction (Davis and Tabor, 1963; Taylor et al., 1978).

Another pathway is the reaction with the amide group of asparagine or glutamine, leading to (hydroxymethyl)-amide derivatives and lysine–amide methylene bridges. It has already been shown that the amide group slowly reacts with formaldehyde to give the hydroxymethyl adduct (Koga et al., 1978; Tome et al., 1981). For the lysine–amide methylene bridge, the primary reaction leads to the formation of a (hydroxymethyl)amine on the lysine side chain which subsequently attacks the amide group (Fraenkel-Conrat and Olcott, 1948a; Shao, 1970; Koga et al., 1978).

The acid-resistant methylene bridges between lysine and tyrosine are assumed to be formed through a Mannich reaction (Short and Ours, 1975; Tome et al., 1984). This type of reaction consists in the condensation of a primary or secondary amine with formaldehyde and a compound containing hydrogen atom of high reactivity; the active hydrogen is replaced by an aminomethyl or a substituted aminomethyl group (Shao, 1970). These mechanisms lead to the observed mono- and disubstitution on lysine nitrogen (Tome et al., 1984).

The last identified pathway is the formation of methyl- and formyllysine. As already proposed from model mixtures analysis (Tyihak et al., 1980; Trezl et al., 1983), formaldehyde present in the medium may reduce the lysine *N*-hydroxymethyl group to an *N*-methyl group while being oxidized itself to formic acid. This formic acid can react with the amino group leading to formyllysine. The *N*-methyl group may also be subjected to another hydroxymethylation and reduction leading to the *N,N*-dimethyl group (Tome et al., 1982). These hypotheses are supported by the formation of formic acid observed in the reaction.

CONCLUSION

^{13}C-Enriched formaldehyde and NMR spectroscopy were useful to identify the reaction products of formaldehyde with BSA. Although the mechanistic description of the reactions is not complete, results clearly demonstrate the great importance of lysine, in conjunction with arginine, asparagine, glutamine, and tyrosine residues in these reactions. Other reactions that do not imply lysine are also assumed with tyrosine, tryptophane, histidine, asparagine, and glutamine; they seem unfavored with BSA but might become important at low lysine levels. More experiments are needed to explain more completely all the mechanisms involved with BSA and other proteins.

Registry No. Methyllysine, 1188-07-4; formyllysine, 1190-48-3; formaldehyde, 50-00-0; *N,N*-dimethyllysine, 2259-86-1; *N*-(hydroxymethyl)-*N*-methyllysine, 94844-32-3.

LITERATURE CITED

Bizzini, B.; Raynaud, M. *Biochimie* **1974**, *56*, 297.
Caldwell, J. B.; Millighan, B. *Text. Res. J.* **1972**, *42*, 122.
Crestfield, A. M.; Moore, S.; Stein, W. H. *J. Biol. Chem.* **1963**, 622.
Davis, P.; Tabor, B. E. *J. Polym. Sci., Part A* **1963**, *A1*, 799.
De Breet, A. J. J.; Dankelman, W.; Huysmans, W. G. B.; De Wit, J. *Angew. Makromol. Chem.* **1977**, *62*, 7.
Dewar, M. K.; Johns, R. B.; Kelly, D. P.; Yates, F. J. *Aust. J. Chem.* **1975**, *28*, 917.
Dunlop, P.; Marini, M.; Fales, H. M.; Sokoloski, E.; Martin, C. J. *Bioorg. Chem.* **1973**, *2*, 235.
Feeney, R. E.; Blankenhorn, G.; Dixon, H. B. F. *Adv. Protein Chem.* **1975**, *29*, 135.
Ferguson, K. A.; Hemsley, J. A.; Reis, P. J. *Aust. J. Sci.* **1967**, 215.
Fraenkel-Conrat, H.; Cooper, M.; Olcott, H. S. *J. Am. Chem. Soc.* **1945**, *67*, 950.
Fraenkel-Conrat, H.; Brandon, B. A.; Olcott, H. S. *J. Biol. Chem.* **1947**, 168, 99.
Fraenkel-Conrat, H.; Olcott, H. S. *J. Am. Chem. Soc.* **1948a**, *70*, 2673.
Fraenkel-Conrat, H.; Olcott, H. S. *J. Biol. Chem.* **1948b**, *174*, 827.
French, I.; Edsall, J. T. *Adv. Protein Chem.* **1945**, *2*, 277.
Galembeck, F.; Ryan, J. R.; Feeney, R. E. *J. Agric. Food Chem.* **1977**, *25*, 238.
Grisolia, S.; Guerri, C.; Godfrey, W. *Biochem. Biophys. Res. Commun.* **1975**, *66*, 1112.
Gustavson, K. H. "The Chemistry of Tanning Processes"; Academic Press: New York, 1956.
Hopwood, D. *Histochimie* **1969**, *17*, 151.
Jentoft, J. E.; Jentoft, N.; Gerken, T. A.; Dearborn, D. G. *J. Biol. Chem.* **1979**, *254*, 4366.
Kelly, D. P.; Dewar, M. K.; Johns, R. B.; Shao, W.; Yates, J. T. In "Protein Cross-Linking"; Friedman, M., Ed.; Plenum Press: New York and London, 1977; pp 641–647.
Kitamoto, Y.; Maeda, H. *J. Biochem. (Tokyo)* **1980**, *87*, 1519.
Koga, D.; Imoto, T.; Nakamura, Y.; Fujikawa, S.; Yagishita, K. *Agric. Biol. Chem.* **1978**, *42*, 1147.
Means, J. E.; Feeney, R. E. *Biochemistry* **1968**, *7*, 2192.
Myers, J. S.; Hardman, J. K. *J. Biol. Chem.* **1971**, *246*, 3863.
Nakai, T.; Ohta, T.; Hayase, N. *Agric. Biol. Chem.* **1979**, *43*, 1179.
Naulet, N.; Tome, D.; Martin, G. J. *Org. Magn. Reson.* **1983**, *21*, 564.
Naulet, N.; Tome, D. *J. Chim. Phys. Phys—Chim. Biol.* **1984**, *81*, 173.
Reis, P. J.; Tunks, D. A. *Aust. J. Biol. Sci.* **1973**, *26*, 1127.
Scott, T. W.; Cook, L. J.; Mills, S. C. *J. Am. Oil Chem. Soc.* **1971**, *48*, 358.
Shao, W. PhD thesis. University of Melbourne, Australia, 1970.
Short, J. H.; Ours, C. W. *J. Heterocycl. Chem.* **1975**, *12*, 869.
Taylor, S. K.; Davidson, F.; Ovenall, W. *Photogr. Sci. Eng.* **1978**, *22*, 134.
Tome, D.; Bertrand, D.; Viroben, G.; Delort-Laval, J. *Ann. Technol. Agric.* **1979**, *28*, 299.
Tome, D.; Naulet, N. *Int. J. Pept. Protein Res.* **1981**, *17*, 501.
Tome, D.; Naulet, N.; Martin, G. J. *J. Chim. Phys. Phys—Chim. Biol.* **1982**, *79*, 361.

Tome, D.; Naulet, N.; Kozlowski, A. *Int. J. Pept. Protein Res.*, in press.
Trezl, L.; Rusnak, I.; Tyihak, E.; Szarvas, T.; Szende, B. *Biochem. J.* **1983**, *214*, 289.
Tyihak, E.; Trezl, L.; Rusnak, I. *Pharmazie* **1980**, *35*, 18.
Walker, J. F. "Formaldehyde"; Krieger, R. E., Publishing Co.: Huntington, NY, 1964.
Wrenn, T. R.; Weyant, J. R.; Wood, D. L.; Bitman, J. *J. Dairy Sci.* **1975**, *59*, 627.
Zelter, S. L.; Leroy, F.; Tissier, J. P. *Ann. Biol. Anim., Biochim., Biophys.* **1970**, *10*, 111.

Received for review July 6, 1984. Accepted December 26, 1984.

Fate of Aldicarb, Aldicarb Sulfoxide, and Aldicarb Sulfone in Floridan Groundwater

Carl J. Miles* and Joseph J. Delfino

The fate of aldicarb, aldicarb sulfoxide, and aldicarb sulfone in Floridan groundwater microcosms was determined. One reaction mechanism observed was base hydrolysis and degradation rates decreased in the order sulfone > sulfoxide >> aldicarb. Appearance of oximes followed the disappearance of corresponding parent compounds while appearance of nitriles was minor and rarely observed. Microcosms amended with crushed limestone showed rates of hydrolysis that were 4–5 times slower than microcosms without limestone. Oxidation of aldicarb to the sulfoxide was minimal within 70 days whereas in separate experiments, the reduction of the sulfoxide to aldicarb was significant over the same time period. The sorption of aldicarb, aldicarb sulfoxide, and aldicarb sulfone onto limestone was not observed.

Recent detection of aldicarb (2-methyl-2-(methylthio)-propionaldehyde *O*-(methylcarbamoyl)oxime, Temik) in Florida groundwater related to its use in citrus agriculture has renewed interest in the fate of this pesticide in the subsurface environment. Although there has been considerable study on the degradation of aldicarb in soils (Coppedge et al., 1967, 1977; Bull et al., 1970; Andrawes et al., 1971; Smelt et al., 1978a, 1978b, 1978c), similar research for natural water matrices is lacking. In Florida, porous, sandy, low organic soils allow rapid penetration of water soluble materials into aquifers where physicochemical conditions differ from most surface waters (i.e., lower temperatures, absence of light, lack of oxygen, and potentially active surfaces).

Several studies have shown that the major metabolic pathway of aldicarb (AS) in soils is rapid oxidation of the parent compound to aldicarb sulfoxide (ASO) followed by slower oxidation to aldicarb sulfone (ASO_2) or hydrolysis to ASO oxime (Figure 1) (Union Carbide, 1983). Additional work has suggested that ASO oxime is degraded further to the corresponding nitrile (Coppedge et al., 1967; Andrawes et al., 1971). Since all of the carbamoyl oximes (AS, ASO, and ASO_2, sometimes called total toxic residue) have a high mammalian toxicity, the hydrolysis step represents a major detoxification mechanism.

Recently, several studies have discussed the degradation of AS, ASO, and ASO_2 in abiotic, reagent grade water solutions (Chapmen and Cole, 1982; Lemley and Zhong, 1983, 1984; Porter et al., 1984; Hansen and Spiegel, 1983). These investigators reported that base hydrolysis is an important reaction mechanism in water and that acid hydrolysis occurs at a lower rate. Most of these authors found that pseudo-first-order kinetics described degradation; experiments with other carbamate pesticides showed that cleavage of the carbamate ester bond is first order with respect to hydroxide and pesticide concentration (Aly and El-Dib, 1972). Studies on the effect of pH on hydrolysis rate allowed calculation of second-order rate constants which showed that base hydrolysis reaction rates decreased in the order ASO_2 > ASO >> AS (Lemley and Zhong, 1983; Porter et al., 1984). Degradation of ASO and ASO_2 was significantly affected by temperature and the reaction followed the Arrhenius relationship. Also, several investigators have reported that increased ionic strength significantly decreases the hydrolysis rate of carbamate pesticides (Fukuto et al., 1967; Aly and El-Dib, 1972; Lemley and Zhong, 1983).

Trehy et al. (1984) investigated the degradation of AS in aerobic and anaerobic Floridan groundwater microcosms, some of which were enriched with microorganisms or limestone. In the presence of high concentrations of microorganisms (pH 6.8) or in the presence of ground limestone (pH 7–7.4), they found that AS degraded rapidly to AS nitrile under anaerobic conditions. In anaerobic groundwater (pH 8.2), AS degraded to AS oxime. These degradation products accounted for a large part (ca. 80%) of the degraded AS. These results contrast with AS degradation rates found by others but can be ascribed to the high concentrations of microbes. The Trehy et al. (1984) investigation demonstrates the importance of naturally occurring factors (i.e., limestone, microorganisms, H_2S, etc.) on AS degradation.

Most of the aforementioned investigators, with the exception of Trehy et al., reported that base hydrolysis of carbamoyl oxime was the major mechanism without ever measuring the actual appearance of the resulting oxime. In short, no pesticide speciation measurements were performed. Soil studies have shown that oxidation is a major metabolic pathway for AS and ASO, suggesting that similar processes should occur in aerobic natural waters. Speciation of AS oxidation products by a gas chromatography (GC) method, not mentioned heretofore (Maitlen et al., 1968), requires an additional liquid chromatograpy (LC) fractionation which apparently was not performed in previous studies. Recently, we developed a high performance liquid chromatography (HPLC) method capable of measuring AS, ASO, ASO_2 and their corresponding oximes and nitriles in groundwater at submilligram per

Department of Environmental Engineering Sciences, University of Florida, Gainesville, Florida 32611.

0021-8561/85/1433-0455$01.50/0