Oxidation of Amino Acids by Dimethyl Sulfoxide

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Dimethyl sulfoxide (DMSO) traces, if present during the hydrolysis of proteins with 6 *N* HCl, cause an oxidative loss of methionine, cystine, and tyrosine with formation of methionine sulfoxide, cysteic acid, and chlorotyrosine as recognizable products. DMSO is at the same time reduced to dimethyl sulfide. Phenol protects the tyrosine but not the sulfur amino acids. To avoid errors in amino acid analysis in protein studies with DMSO, great care must be taken to remove all traces of DMSO. Similar to DMSO, methionine sulfoxide causes an oxidative loss of cystine and tyrosine during hydrolysis.

During a study of chemical methods which may be useful for determining the "nutritionally available" methionine in foods (Lipton and Bodwell, 1972), a loss of tyrosine, methionine, and cystine was observed in lyophilized meat samples which had been treated at room temperature with aqueous dimethyl sulfoxide (DMSO) prior to acid hydrolysis for amino acid analysis. Since DMSO is widely used in biochemical studies which may involve amino acid analysis (Friedman, 1968; Jacob *et al.*, 1971; Leake, 1967) and is in fact used as a solvent in a ninhydrin reagent (Moore, 1968), this observation seemed to be of sufficient general interest as to merit further study. Although DMSO has been used primarily as a solvent which remarkably accelerates many organic reactions (Fieser and Fieser, 1967; Kharasch and Thyagarajan, 1966; Martin *et al.*, 1967; Parker, 1965) it has also found considerable use as a mild oxidant (Durst, 1969; Epstein and Sweat, 1967). This oxidizing property of DMSO was responsible for the alteration of the amino acids described in this paper, which also relates the DMSO effect to the oxidative losses of amino acids which are frequently observed during the hydrolysis of proteins.

MATERIALS AND METHODS

Chemicals and Sources. Dimethyl sulfoxide (DMSO), reagent grade, Eastman Kodak Co.; *dl*-methionine sulfoxide and cysteic acid, Calbiochem Co.; amino acid Calibration Mixture Type I, 2.5 μmol/ml, Beckman Instruments; cytochrome c, Type III, from horse heart, Sigma Chemical Co.; trypsin, bovine pancreas, 207 μg/mg, Worthington Biochemical Corp. Constant boiling HCl was prepared by dilution and distillation of Baker's analyzed reagent-grade acid.

Amino Acid Analyses. A Phoenix Biolyzer Model 3000 was used for amino acid analyses as previously indicated (Lipton *et al.*, 1971) for the acidic and neutral amino acids, except that the change to 4.26 buffer was at an elution volume of about 130 ml. Basic amino acids were also run by the procedure of Spackman *et al.* (1958) using a 17-cm column of cation exchange resin and a pH of 5.25, 0.35 *M* citrate buffer. The Brij-35 detergent previously used in all buffers was omitted.

Acid Hydrolysis Procedure. Hydrolysis was in constant boiling (6 *N*) hydrochloric acid for 22 hr at 110°, using either evacuated 12-ml tubes or 50-ml round-bottomed flasks. Evacuation was with an oil pump as described by Moore and Stein (1963), and hydrolysis vessels were sealed by closing the stopcocks in attached ground glass tube adapters.

DMSO Treatments. About 20 mg of the lyophilized meat sample was weighed, transferred into a 50-ml round-bottomed flask, and suspended in 0.2 ml of water and 1.8 ml of DMSO. The sample was kept at 24° for an overnight period of about 20 hr and was then evaporated to dryness at room temperature with the aid of an oil vacuum pump. After the addition of 5 ml of 6 *N* HCl, hydrolysis was then carried out by the procedure described above. Purified proteins were treated with DMSO in a similar manner using one-half the quantities described above. Amino acid standard solutions were evaporated *in vacuo* to dryness, and the residues were then taken up either in 6 *N* HCl or in buffers to which various levels of DMSO were added. Samples of the standards dissolved in acid were then heated as described above and those dissolved in buffers were analyzed without heating.

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RESULTS AND DISCUSSION

Initially, lyophilized pork samples were allowed to stand overnight at room temperature in 90% aqueous DMSO solution and were then dried *in vacuo* and hydrolyzed in 6 *N* HCl solution for amino acid analysis. As seen by a comparison of chromatograms A and B of Figure 1, tyrosine was completely eliminated from the treated meat sample and methionine was largely oxidized to the sulfoxide peaks (at *ca.* 42 min) which just precede aspartic acid. Moreover, the appearance in the DMSO-treated sample of the peak at about 18 min due to cysteic acid was evidence that cystine also had been partly oxidized. Authentic methionine sulfoxide and cysteic acid samples were chromatographed on the same column and were found to coincide with the peaks respectively ascribed to these compounds in the DMSO-treated samples. In the region of the basic amino acids, not illustrated in the figure, a peak at 25 min which just preceded lysine was present in the treated sample and corresponded with chlorotyrosine (Sanger and Thompson, 1963). Following these observations with meat samples, the purified proteins cytochrome c and trypsin were similarly exposed to 90% DMSO prior to hydrolysis for amino acid analysis. As seen in chromatograms C–F of the figure, the tyrosine contents of the treated samples were much lower than those of the untreated hydrolyzed proteins. This is particularly evident for the comparatively tyrosine-rich trypsin sample (chromatogram F). In addition, alterations of sulfur amino acids were evident.

The possibility was first considered that the DMSO might be oxidizing the amino acids independently of the hydrolysis step. A standard amino acid calibration mixture was therefore evaporated *in vacuo* to dryness, and was allowed to stand overnight at room temperature in 90% aqueous DMSO, but there was no alteration of the amino acids without the hydrolysis step. However, when the DMSO-treated standard amino acid mixture was reevaporated to dryness and heated for 22 hr in 6 *N* HCl, extensive destruction of tyrosine and the sulfur amino acids was evident in the amino acid chromatograms. Losses were similar to those shown in chromatogram B. Although evaporation *in vacuo* for removal of the DMSO

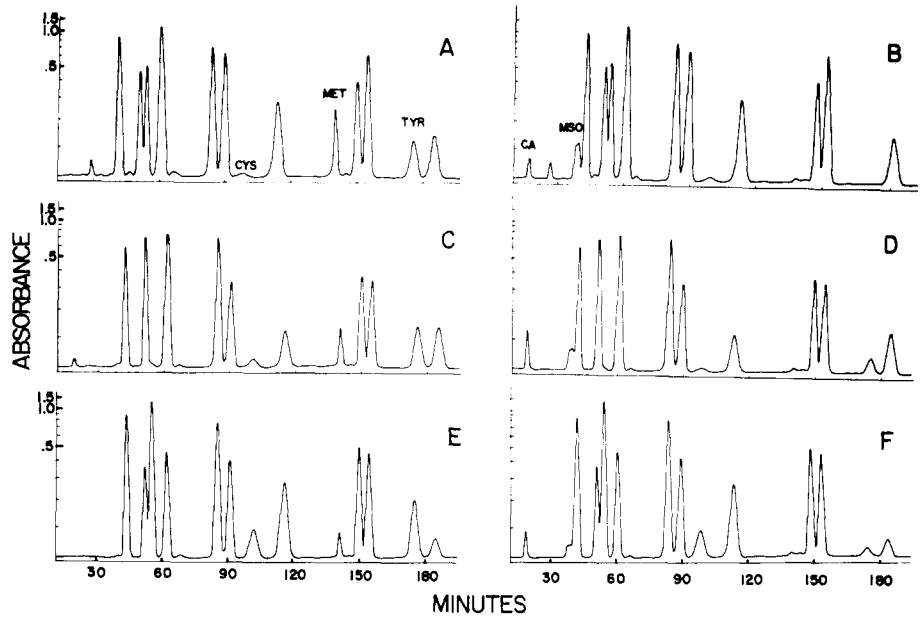

Figure 1. Chromatograms of acidic and neutral amino acids of acid-hydrolyzed samples. Curve A, 1.0 mg of untreated lyophilized pork; Curve B, 1.0 mg of DMSO-treated lyophilized pork; Curve C, 0.5 mg of untreated cytochrome c; Curve D, 0.5 mg of DMSO-treated cytochrome c; Curve E, 0.5 mg of untreated trypsin; Curve F, 0.5 mg of DMSO-treated trypsin. Abbreviations: CA, cysteic acid; MSO, methionine sulfoxide.

from the treated samples was sufficient to remove also most of the ammonium salt of the samples, apparently DMSO traces still persisted. However, prolonged high vacuum drying for several hours with care to avoid reintroduction of traces of DMSO prior to acid hydrolysis eliminated the oxidative losses of the sensitive amino acids. Moreover, in this study the addition of as little as 0.01% of DMSO to the 6 *N* HCl immediately prior to heating a standard amino acid mixture at 110° *in vacuo* for 22 hr caused destruction of tyrosine and the sulfur amino acids. The oxidative losses were due to the combined presence of DMSO and 6 *N* HCl during the hydrolysis procedure. The formation of dimethyl sulfide by reduction of the DMSO was indicated by its strong characteristic odor.

The addition to the 6 *N* HCl of a small crystal of phenol prior to hydrolysis of the protein protected tyrosine from the DMSO, but oxidation of the sulfur amino acids still occurred. This observation is consistent with a halogenation mechanism for the tyrosine destruction. Sanger and Thompson (1963) have previously suggested the addition of phenol to prevent the formation of halotyrosines from traces of free halogens formed during the hydrolysis of proteins. Our chromatographic separations did not permit a positive identification of all of the various possible halotyrosines. [Among those which may be formed (Sanger and Thompson, 1963) during hydrolysis in 6 *N* HCl were mono and di derivatives of both bromo (from an HBr impurity in the HCl) and chloro compounds and mixed bromo-chloro derivatives.] The halotyrosine (25-min peak in the basic region) quantitatively accounted for only a small fraction (*ca.* 5–10%) of the tyrosine which was destroyed.

There are several indications that the oxidation of amino acids by DMSO may have a practical analytical significance. Although DMSO is not as abundant a constituent of foods as its reduction product dimethyl sulfide, DMSO nevertheless has been observed to be a trace constituent of stale nonfat dry milk (Ferretti and Flanagan, 1972). Of probably greater significance, however, is the occurrence of methionine sulfoxide as a common constituent of biological materials. While this sulfoxide is not a proven constituent of native proteins, it is known to be readily formed by air oxidation of methionine residues of proteins. Acid hydrolysis of proteins containing methionine sulfoxide has previously been reported (Neumann *et al.*, 1962) to be accompanied by losses of cystine and tyrosine. In our study, the addition of methionine sulfoxide like that of DMSO to a standard mixture of free amino acids caused an oxidative loss of cystine and tyrosine under acidic conditions used for protein hydrolysis. Previous studies have variously stated that methionine sulfoxide reverts largely to methionine (Ray and Koshland, 1962), is converted to a 50:50 mixture of free methionine and its sulfoxide (Njaa, 1962), gives free methionine as well as several methionine derivatives (Floyd *et al.*, 1963), and partly or entirely survives hydrolysis unchanged (Doscher and Hirs, 1967). Mercaptoethanol has been employed during acid hydrolysis for reduction of methionine sulfoxide in order to improve the recovery of methionine (Keutmann and Potts, 1969). In addition, mercaptoethanol also served to improve the recovery of tyrosine. In the absence of added reducing agents, the amino acids cystine and tyrosine (and probably tryptophan as well) may serve as sulfoxide reductants during 6 *N* HCl hydrolysis, and thus the fate of methionine sulfoxide is determined by the accompanying amino acid residues.

Krull and Friedman (1967) have reported an inability to completely remove DMSO at reduced pressures, and we have also found it to be difficult. However, we have noted no published warning that these persisting traces of DMSO may cause an analytical loss of sulfur amino acids and tyrosine, if present during protein hydrolysis. [Spencer and Wold (1969) employed comparatively high (0.2–0.3 *M*) levels of DMSO during acid hydrolysis for the oxidative conversion of cysteine and cystine to cysteic acid. Under these conditions, losses of methionine, tyrosine, and histidine were also observed. Earlier references to the oxidation of thiols and sulfides by DMSO have been reviewed by Epstein and Sweat (1967) and did not include an S–S bond cleavage and oxidation to SO_3^-.] Although we have not quantitatively accounted for the amino acid losses in terms of the products formed, the striking losses observed suggest that caution must be exercised when conducting amino acid analyses on protein samples previously treated with DMSO.

ACKNOWLEDGMENT

We thank Dr. J. S. Wall for helpful discussion of the manuscript and for bringing to our attention the paper of Spencer and Wold (1969).

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Received for review August 17, 1972. Accepted November 10, 1972.

Factors Influencing the Rate of Formation of Nitrosomorpholine from Morpholine and Nitrite: Acceleration by Thiocyanate and Other Anions

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The kinetics of nitrosation of morpholine, a weak base and possible food contaminant, have been investigated. Temperature, pH, and composition of the reaction medium have been explored as experimental variables, and certain anions have been shown to influence strongly the reaction rate. The extent of the effect of thiocyanate, a strong promoter of nitrosation, is dependent upon pH and relative reactant concentrations. pH-Independent rate constants have been calculated for two nitrosation mechanisms, and mathematical expressions have been derived which accurately predict overall rates of nitrosation.

The toxicity and carcinogenicity of nitrosamines are well known, and the subject has recently been reviewed (Lijinsky and Epstein, 1970; Wolff and Wasserman, 1972). Morpholine is a particularly interesting compound for the study of nitrosation reactions for a variety of reasons, including the fact that as a weak base it will nitrosate very rapidly compared to compounds like dimethylamine. Morpholine is also a potential inadvertent food contaminant, since it is a permitted corrosion inhibitor in steam boilers. Also, a variety of substituted morpholine structures are naturally occurring compounds. Finally, *N*-nitrosomorpholine is a known carcinogen for the rat (Druckrey *et al.*, 1967).

Previous investigations on nitrosation kinetics (Mirvish, 1970, 1972) have only briefly considered morpholine, and the accelerating influence of thiocyanate on morpholine nitrosation was first demonstrated by Boyland (1972) and Boyland *et al.* (1971). This latter investigation neglected the influence of pH on reaction rate. In this paper, pH-independent rate constants are calculated for morpholine nitrosation under a variety of reaction conditions, and the temperature-dependency of the reaction is also analyzed.

MATERIALS AND METHODS

All chemicals were reagent grade. Solutions of amine and sodium nitrite were separately adjusted to the reaction pH with perchloric acid and mixed immediately prior to incubation. Anions were used as their potassium salts and were preincubated with the amine solution. After initiation of the reaction, samples were taken at fixed intervals and quenched by the immediate addition of a large excess of ammonium sulfamate. At reaction pH's above 2, it was necessary to heat the solution to 65° for 5 min after ammonium sulfamate addition. When the pH was above 3.5, perchloric acid was added to reduce the pH to below 3.0. A zero time sample, to which ammonium sulfamate was added immediately following the mixing of nitrite and morpholine, was included in each kinetic run.

The concentration of *N*-nitrosomorpholine (NM) was measured spectrophotometrically at 260 nm using an absorbance of 3540 l. cm^{-1} mol^{-1}. Although λ_{max} for NM is at 235 nm, the substantial absorption of the parent amine at this wavelength makes it simpler to use 260 nm. NM solutions obey the Lambert-Beer law at this latter wavelength over the concentration range studied. Nitrite was measured using the automatic procedure of Fan and Tannenbaum (1971).

Rate constants were determined from initial rates, since nitrite concentrations in open vessels are inconstant upon prolonged incubation (Table I). The method used was the Method of Initial Rates: the NM concentration was plotted as a function of time, the best curve fitted with a French Curve, and the best straight line fitted with a straight edge between the origin (t = 0, NM = 0) and the French Curve. This straight line was the tangent to the curve, and its slope was the initial reaction rate. A typical run had the following reaction conditions: nitrite and morpholine were each 5 to 10 mM, temperature held constant at 25°, and the reaction was followed for 30 to 60 min. The yield of nitrosomorpholine varied from 0.5 to 5% of the theoretical maximum.

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