Table V. Comparison of HOO Energy Levels and Fluorescence Quenching Constants

	ROCH=CHOR	CH_2=C(OR)$_2$	CH_2=CHOR
E_{HOO}[a]	$\alpha + 0.47\beta$	$\alpha + 0.58\beta$	$\alpha + 0.73\beta$
k_q[b]	2.5×10^9	1.1×10^9	$<5 \times 10^8$

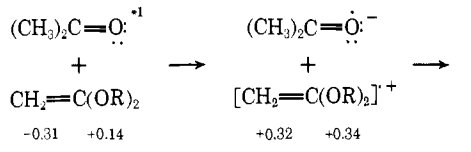
[a] Energy of highest occupied orbital. [b] Rate constant for quenching of acetone fluorescence, M^{-1} sec^{-1}.

state acetone and ethylene or (b) oxetane) is strongly corroborated by the new data reported here. However, details of the mechanism suggested for addition of acetone singlets to enol ethers[4,8] (direct addition of acetone singlets to the enol ether to form a biradical intermediate) should be modified in light of the results reported here and in other recent reports.[9-12] Thus, direct formation of a biradical intermediate from singlet acetone and an enol ether is not consonant with the relative reactivities reported in Table II, since on this basis, 1,1-diethoxyethene would be expected to be considerably more reactive than DEE. Furthermore, in order to explain the poor regiospecificity of oxetane formation from enol ethers, either preferred dissociation (relative to oxetane formation) of the "best" biradical or some other factor must be brought into consideration.

Caldwell[9-11] has suggested that a precursor to the biradical preceding oxetane formation from benzophenone triplets and ethylenes may explain certain isotope effects. Similar conclusions were reached from consideration of the reactivities of quenching of butyrophenone triplets by a series of ethylenes.[12] Our results are consistent with a similar attitude toward the reaction of alkanone singlets with enol ethers. Thus, a biradical intermediate is attractive in order to explain the loss of stereochemistry in oxetane formation and concurrent cis-trans isomerization when alkanone singlets interact with ethylenes.

The evidence presented here strongly supports a major charge-transfer interaction contribution to the transition state for quenching of alkanone singlets by enol ethers. Furthermore, the same features, if operating in a product (or biradical) producing step sequential to the quenching step, suggest a possible source of the poor regiospecificity of oxetane formation. Scheme I indicates that although the ground states of

Scheme I. Comparison of Charge Distributions at the Ethylenic Carbons for the Ground-State and Radical Cation of a 1,1-Dialkoxyethylene



enol ethers (*e.g.*, 1,1-dialkoxyethylenes) are strongly polarized so that C-2 has considerable negative charge characteristics, the radical cation possesses surprisingly comparable positive charge characteristics at both ethylenic carbons. Thus, collapse of the radical cation-radical anion pair to biradicals might well be nonselective and may not reflect biradical stability. A "free" charged radical pair need not exist, but only the transition state for partitioning to biradicals need "feel" the coulombic influences suggested by the charge densities given in Scheme I. It should be noted, however, that lack of regiospecificity can also be explained by (1) reversibility arguments (*i.e.*, addition to the 2-carbon atom of CH_2=C(OCH$_3$)$_2$ may be favored over addition to the 1-carbon, but the former addition may be more reversible[13] than the latter addition) and (2) "hot" or indiscriminant intermediates.

In summary, although (1) the rates of alkanone fluorescence quenching by unsaturated nitriles parallel LUO energies and (2) the rates of alkanone fluorescence quenching by enol ethers parallel HOO energies, the regiospecificities of products which result from these interactions are determined by other mechanistic factors. In the case of unsaturated nitriles, stereospecific and regiospecific collapse of a singlet exciplex seems likely, while for enol ethers initial attack of alkanone singlets on the π cloud, rather than a specific carbon, seems likely. The latter process is then followed by product formation from a transition state (or intermediates) possessing strong charge-transfer characteristics.

(8) N. J. Turro and P. A. Wriede, *J. Amer. Chem. Soc.*, 90, 6863 (1968).
(9) R. A. Caldwell, *ibid.*, 92, 1439 (1970).
(10) R. A. Caldwell and S. P. James, *ibid.*, 91, 5184 (1969).
(11) R. A. Caldwell and G. W. Sovocool, *ibid.*, 90, 7138 (1968).
(12) I. H. Kochevar and P. J. Wagner, *ibid.*, 92, 5742 (1970).
(13) In other words, collapse of a radical cation-radical anion pair might lead preferentially to the most stable biradical, which then also collapses preferentially. The low total quantum yields of oxetanes (~0.1) under the conditions reported here allow entertainment of such a proposal.
(14) National Institutes of Health Predoctoral Fellow, 1970-present.

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Reduction of Disulfide Bonds in Peptides and Proteins by Dithiothreitol in Liquid Ammonia[1,2]

Sir:

Disulfide bonds in peptides and proteins were completely and rapidly reduced by dithiothreitol (DTT)[3] in liquid ammonia[4] without apparent side reactions.[5] The resulting thiol groups were selectively alkylated by alkyl chlorides added to the liquid ammonia solutions.[6,7] Essentially homogeneous *S*-alkylated derivatives were isolated after evaporation of the ammonia

(1) Supported by Public Health Service Research Grants C-6516 from the National Cancer Institute and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.
(2) Abbreviation: DTT, dithiothreitol.
(3) W. W. Cleland, *Biochemistry*, 3, 480 (1964).
(4) In aqueous solution high concentrations of denaturants are often necessary to assure full disulfide reduction [J. L. Bailey, "Techniques in Protein Chemistry," Elsevier, New York, N. Y., 1967, pp 115-124; T. A. Bewley and C. H. Li, *Int. J. Protein Res.*, 1, 117 (1969)].
(5) Reduction of disulfide bonds by sodium in liquid ammonia [V. du Vigneaud, L. F. Audrieth, and H. S. Loring, *J. Amer. Chem. Soc.*, 52, 4500 (1930)] can give peptide bond cleavage [K. Hofmann and H. Yajima, *ibid.*, 83, 2289 (1961); M. Wilcheck, S. Sarid, and A. Patchornik, *Biochim. Biophys. Acta*, 104, 616 (1965); W. F. Benisek and R. D. Cole, *Biochem. Biophys. Res. Commun.*, 20, 655 (1965); W. F. Benisek, M. A. Raftery, and R. D. Cole, *Biochemistry*, 6, 3780 (1967)].
(6) V. du Vigneaud, L. F. Audrieth, and H. S. Loring, *J. Amer. Chem. Soc.*, 52, 4500 (1930).
(7) M. D. Armstrong and J. D. Lewis, *J. Org. Chem.*, 16, 749 (1951).

and simple repeated washing of the remaining solids with methanol which completely removed excess reagents and by-products.[8]

For a typical experiment insulin (92 mg) was dissolved in dry liquid ammonia (300 ml) which was stirred under nitrogen and protected from moisture and CO_2. Dithiothreitol (148 mg, corresponding to 20 mol of DTT/mol of disulfide) was added. The solution was kept at the boiling point (at about −33°, atmospheric pressure) for 1 hr. Chloromethane gas was then introduced until the thiols were fully methylated (after 1–3 min). Complete *S*-alkylation was ascertained by a negative nitroprusside reaction directly in the liquid ammonia solution[6] of aliquots. The liquid ammonia evaporated spontaneously. The vacuum-dried residue was triturated with methanol (15 ml), centrifuged, and washed three–four times with methanol (10 ml each) and finally with peroxide-free ether, followed by centrifugation. A white powder was obtained after drying (95 mg, 98%, mixture of tetra-*S*-methyl A chain and bis-*S*-methyl B chain[9]). In other preparations the methanol-wet pellet was dissolved in 0.1 *N* ammonium hydroxide or in 50% acetic acid and lyophilized. Amino acid analysis[10] gave: *S*-methylcysteine 6.2, no trace of cysteine or cystine, and values for all other amino acids (based on Ala, 3.0) which agreed very closely with the theory.

The above example shows the efficiency and convenience of the procedure, because (a) complete and selective *S*-methylation of peptides and proteins has until recently been unattainable,[11] (b) complete reduction and *S*-alkylation of insulin previously has been difficult,[4] and (c) salt-free, fully *S*-alkylated protein derivatives are obtained within 1 day in close to quantitative yields. Sodium borohydride, mercaptoethanol, and dithiothreitol were examined for their usefulness in the liquid ammonia procedure. Dithiothreitol gave the best results. A 20-fold molar excess of DTT per mole of disulfide was generally sufficient. To assure full conversion of disulfides to thiols, 1-hr reduction periods were usually applied.

Thiol alkylation was best achieved by addition of alkyl chlorides directly to the liquid ammonia solution.[6,7] Alkyl iodides and bromides, commonly used for *S*-alkylation in aqueous solution,[4] reacted to considerable degrees with liquid ammonia, but alkyl chlorides appeared to be practically inert under the conditions employed in this procedure.[12] To assure complete *S*-alkylation, a 20-fold molar excess of alkyl chloride over dithiothreitol was used. Liquid alkylating agents (*e.g.*, benzyl chloride) were conveniently added by pipet, solid reagents (chloroacetic acid, chloroacetamide, β-chloroethylamine hydrochloride) through a powder funnel. The following products were prepared in 90–98% yields essentially as described above:[13] (bis-*S*-methyl)lysine-vasopressin [*S*-methylcysteine, 2.1 (2)]; (hexa-*S*-benzyl)insulin [*S*-benzylcysteine, 5.9 (6)]; (tetra-*S*-benzyl)neocarzinostatin[14] [*S*-benzylcysteine, 3.8 (4)]; (tetra-*S*-carboxymethyl)-neocarzinostatin [*S*-carboxymethylcysteine, 3.9 (4)]; (octa-*S*-carboxamidomethyl)lysozyme [*S*-carboxymethylcysteine, 7.8 (8)]; (oct-*S*-β-aminoethyl)lysozyme [*S*-β-aminoethylcysteine, 7.6 (8)]. To ascertain the absence of undesired peptide bond cleavage, N-terminal analysis was carried out using 1-dimethylaminonaphthaline-5-sulfonyl chloride.[15] Identical end groups were obtained from starting native proteins and reduced and *S*-alkylated products.

In conclusion, full reduction of disulfide bonds and complete and selective *S*-alkylation of liquid ammonia–soluble peptides and proteins[16] are described. This procedure offers an alternative approach for the modification of proteins possessing disulfides that resist reduction in aqueous solution. Uniform derivatives, potentially useful in many areas of protein chemistry, can be prepared effectively and rapidly. Principal improvements over the previously known sodium in liquid ammonia procedure[5–7] consist in (a) absence of peptide bond scission or other side reactions, and (b) complete removal of reagents and by-products by simple washing with methanol.

(8) Complete removal of buffer salts, denaturants, reducing agents (*e.g.*, $NaBH_4$), and alkylating agents after disulfide reductions in aqueous phase requires laborious procedures, such as dialysis, gel filtration, or ion exchange chromatography; see ref 4.

(9) Thin-layer chromatography (50% acetic acid) of the isolated product gave two spots (A and B chains).

(10) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958); D. H. Spackman, *Methods Enzymol.*, 11, 3 (1967).

(11) The use of methyl *p*-nitrobenzenesulfonate in aqueous solution [R. L. Heinrikson, *Biochem. Biophys. Res. Commun.*, 41, 967 (1970)] still requires time-consuming work-up (dialysis); see ref 4. *S*-Methylated polypeptides are of interest since chemical cleavage at *S*-methylcysteine residues has been described [E. Gross, J. L. Morrell, and P. Q. Lee, *Proc. Int. Congr. Biochem.*, 7*th*, Part XI, 535 (1967); T. F. Spande, B. Witkop, Y. Degani, and A. Patchornik, *Advan. Protein Chem.*, 24, 119 (1970)].

(12) No glycine could be detected by amino acid analysis in a *S*-carboxymethylated product prepared with the use of chloroacetic acid; in contrast, large amounts of glycine resulted from the use of iodoacetic acid. However, no potential side reactions of alkyl iodides with lysine, histidine, methionine, or tryptophan residues (see Bailey, ref 4) were detected by amino acid analysis or by ultraviolet difference spectrum.

(13) Degrees of reduction and alkylation were determined from the amounts of *S*-alkylcysteine (given in brackets, theory in parentheses) in amino acid analyses after total acid hydrolysis.

(14) H. Maeda, K. Kumagai, and N. Ishida, *J. Antibiot., Ser. A*, 19, 253 (1966); J. Meienhofer, H. Maeda, C. B. Glaser, and J. Czombos, "Proceedings of the Second American Peptide Symposium, Cleveland, Ohio, 1970," S. Lande, Ed., Gordon and Breach, New York, N. Y., in press.

(15) W. R. Gray, *Methods Enzymol.*, 11, 139 (1967); K. R. Woods and K. T. Wang, *Biochim. Biophys. Acta*, 133, 369 (1967).

(16) For proteins that do not dissolve in liquid ammonia the procedure should be modified. For instance, human growth hormone and bovine pancreatic ribonuclease A are insoluble. The former was completely reduced in suspension using 1000-fold excess of DTT and then quantitatively benzylated. Alternatively, it was dissolved in a very small volume of water (10 mg in 0.5 ml) and then added to liquid ammonia (100 ml) where it remained in solution. The ribonuclease dissolved slowly in the presence of 20 equiv of DDT within 4–5 hr. Addition of alkylating agents at this point gave 4-*S*-alkylcysteines.

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Trimethylenemethane and the Methylenecyclopropane Rearrangement[1]

Sir:

There has been much interest recently in the methylenecyclopropane rearrangement,[2] and in the nature of trimethylenemethane (**1**) which has been prepared[3] as an observable entity in matrices at low temperatures.

(1) This work was supported by the Air Force Office of Scientific Research through Contract No. F44620-C-70-0121.

(2) For a summary and references, see W. von E. Doering and H. D. Roth, *Tetrahedron*, 26, 2825 (1970).

(3) P. Dowd, *J. Amer. Chem. Soc.*, 88, 2587 (1966); 89, 715 (1967).