other olefins (*e.g.*, 2-butene, cyclohexene) lie outside this simple correlation. The values of K_{eq}, which reflect the superposition of the separate trends of k_1 and k_4, do not exhibit any obviously simple correlation (such as k_1 does) with olefin structure. 3. The oxymercuration equilibrium quotients (K_{eq}) span the range of values 5×10^4–1.2×10^7. The oxymercuration equilibria (eq 1) are thus displaced far to the right even in strongly acid solutions and are correspondingly difficult to measure directly.[8] In the two cases for which K_{eq} has previously been estimated, our values are in satisfactory accord with the earlier ones, namely 5.1×10^4 for Hg(cyclohexene)OH^+,[9] and 3.6×10^6 for Hg(ethylene)OH^+[10] (both at unit ionic strength). 4. Although our measurements do not yield separate values of k_5, k_{-5}, and k_6, it seems likely that for most of the reactions $k_6 \gtrsim k_{-5}$ and hence that $k_5' \sim k_5$.[11] In line with this, the trend of k_5' values for different unsaturated compounds ($O1_B$) are found to be similar to the corresponding trends for the oxymercuration rate constants (k_1^B).[1] The expectation of such a correlation is reasonable since both k_1^B and k_5^B refer to oxymercuration processes for the same olefin. The rather high observed values of the ratio k_1^B/k_5' (10^4–10^7) reflect the much lower reactivities of the oxymercurial ions (compared to the free Hg^{2+} ion) toward further oxymercuration.

Finally, in view of the recent controversies surrounding this subject, it should perhaps be noted that our observation of these olefin exchange reactions and our interpretation of their mechanisms neither provide support for, nor rule out, the occurrence (in concentrations too low to be kinetically detectable from either the acid or olefin dependence) of the mercurinium ions that have been postulated as coexisting in equilibrium with oxymercurials and/or as intermediates in oxymercuration–deoxymercuration reactions.[12]

Acknowledgments. Support of this work by the National Science Foundation and by the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged. J. E. B. also thanks the Shell Companies Foundation, Inc. for a Fellowship award.

(6) L. L. Schaleger, M. A. Turner, T. C. Chamberlin, and M. M. Kreevoy, *J. Org. Chem.*, 27, 3421 (1962).

(7) K. Ichikawa, K. Nishimira, and S. Takayama, *ibid.*, **30**, 1593 (1965).

(8) Deoxymercuration is favored by complexing of Hg^{2+}, notably by halide ions. In the presence of halide ions deoxymercuration can be effected at moderate acid concentrations and several quantitative kinetic studies of such halide-assisted deoxymercuration reactions have been reported.[6,7] While we have noted certain parallels between the rate constants of such reactions and our values of k_4, halide-assisted deoxymercurations do not correspond strictly to the reverse of the oxymercuration reactions depicted in eq 4.

(9) H. Lucas, F. Hepner, and S. Winstein, *J. Amer. Chem. Soc.*, **61**, 3102 (1939).

(10) P. Brandt and O. Plum, *Acta Chem. Scand.*, 7, 97 (1953).

(11) It should be possible to derive actual values of k_5 from kinetic measurements on self-exchange reactions (*i.e.*, $O1_A \equiv O1_B$) using isotopically labeled olefins since for such reactions (apart from small kinetic isotope effects) $k_{-5} = k_6$. Attempted measurements of this type are now in progress.

(12) R. G. Parker and J. D. Roberts, *J. Amer. Chem. Soc.*, **92**, 743 (1970), and references therein.

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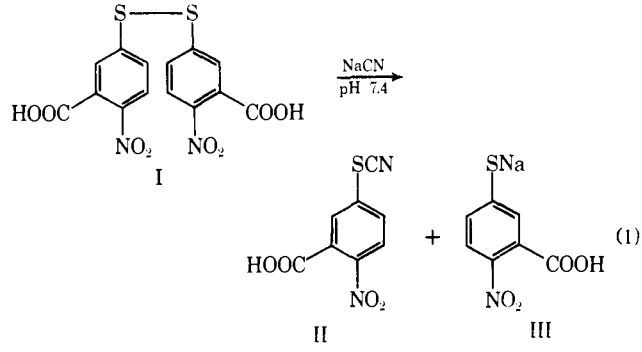
Selective Cyanylation of Sulfhydryl Groups

Sir:

Cysteine peptide bonds can be cleaved *via* a β-elimination reaction of *S*-dinitrophenylcysteine derivatives to dehydroalanine residues.[1,2] In peptide chains containing both cysteine and cystine residues, this method cannot be used since, under the alkaline conditions required for the elimination, cystine residues may also be converted to dehydroalanine residues by elimination of disulfide groups.[3,4] The elimination of the stronger electron-attracting *S*-picryl group is rather more selective, but the yields are lower due to partial alkaline hydrolysis.[4] Cyanide ions are known to slowly split the disulfide group of the cystine residue at pH 7–8 to form a thiol and a β-thiocyanoalanine residue which subsequently undergoes cyclization to an *N*-acyl-2-iminothiazolidine derivative, followed by hydrolysis of the *N*-acyl bond.[5,6] It was realized that if cysteine residues could be cyanylated directly and selectively to β-thiocyanoalanine residues, the cyclization reaction could be used for the cleavage of cysteine peptide bonds in the presence of cystine residues.

We wish to report in the present communication a method for the direct conversion of thiol groups into thiocyanates using 2-nitro-5-thiocyanobenzoic acid (II, NTCB) under very mild conditions. The reagent was synthesized by treatment of 5,5′-dithiobis(2-nitrobenzoic acid) (I, Ellman's reagent,[7] DTNB) with NaCN in equimolar concentrations in 0.1 *M* phosphate buffer, pH 7.4.



The thionitrobenzoate (III, TNB) formed together with NTCB (II) was removed from the product mixture by treatment with bromoacetylcellulose,[8] thus shifting the equilibrium toward quantitative completion of the reaction. The reagent II was purified by crystallization from ethyl acetate–petroleum ether; mp 162–163°. *Anal.* Calcd for $C_8H_4N_2O_4S$: C, 42.87; H, 1.80; N, 12.50; S, 14.28. Found: C, 42.95; H, 1.75; N, 12.45; S, 14.06. The mass spectrum showed the parent molecular peak at *m/e* 224. The uv spectrum of the reagent is shown in Figure 1. ^{14}C-Labeled NTCB was analogously prepared by using $Na^{14}CN$.

(1) A. Patchornik and M. Sokolovsky, *J. Amer. Chem. Soc.*, **86**, 1206 (1964).

(2) M. Sokolovsky, T. Sadeh, and A. Patchornik, *ibid.*, **86**, 1212 (1964).

(3) Z. Bohak, *J. Biol. Chem.*, **239**, 2878 (1964).

(4) T. F. Spande, B. Witkop, Y. Degani, and A. Patchornik, *Advan. Protein Chem.*, **24**, 98 (1970).

(5) J. L. Wood and N. Catsimpoolas, *J. Biol. Chem.*, **238**, 2887 (1963).

(6) N. Catsimpoolas and J. L. Wood, *ibid.*, **241**, 1790 (1966).

(7) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

(8) A. Patchornik, Israel Patent Application No. 18207 (1962).

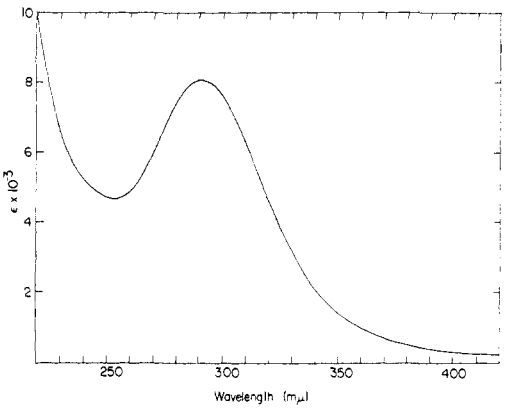


Figure 1. Absorption spectrum of NTCB in 0.1 *M* phosphate buffer, pH 7.4.

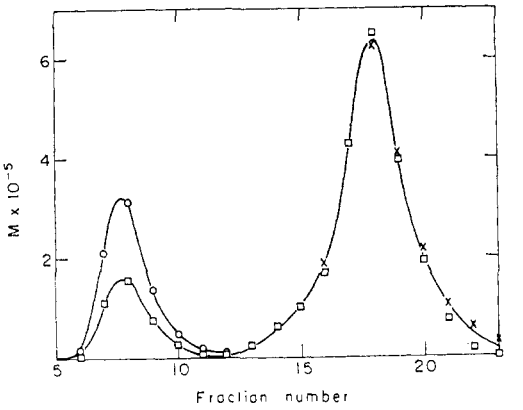
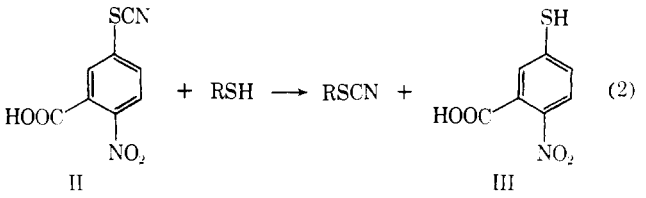


Figure 2. Cyanylation of papain with NTCB. Sephadex G-25 chromatography in 0.1 *M* phosphate buffer, pH 7.4, of the products of the reaction of papain, 0.05 m*M*, with ^{14}C-NTCB, 0.2 m*M*, (212,000 cpm/μmol) in the same buffer: (○) protein, determined by using ϵ_{280} 51,000 [A. N. Glazer and E. L. Smith, *J. Biol. Chem.*, **236**, 2948 (1961)]; (□) carbon-14, determined by liquid scintillation; (×) NTCB, determined by using ϵ_{293} 8000 (see Figure 1).

The reaction of NTCB with sulfhydryl compounds takes place at pH 7–8 at room temperature. The reaction can be followed spectrophotometrically at either 293 mμ, measuring the disappearance of NTCB, or at 412 mμ, measuring the appearance of TNB. In most cases we found it preferable to measure the appearance of TNB.



II III (2)

NTCB reacted with β-mercaptoethanol in 0.1 *M* phosphate buffer, pH 7.4, with a bimolecular rate constant of 3.0 l. mol^{-1} sec^{-1} at 22°. When using ^{14}C-NTCB, the amount of liberated NTB was found to be equivalent to the labeled-carbon content of the reagent. The reaction between 10^{-3} *M* cysteine and an equimolar amount of ^{14}C-NTCB in 0.1 *M* phosphate buffer, pH 8.0, was completed within 3 min. After further incubation of the reaction mixture for 3 hr at 37° and removal of TNB with bromoacetylcellulose, ^{14}C-labeled 2-iminothiazolidine-4-carboxylic acid was found in 90% yield by the paper chromatographic system of Bradham, *et al.*[9] The product was apparently formed from the intermediary β-thiocyanoalanine, which is known to cyclize under similar conditions.[10]

The S-cyanylation reaction was further demonstrated on papain, a protein containing in its active form a single cysteine residue and three disulfide bonds that link six half-cystine residues. The active-enzyme preparation (Worthington lot 9JA, of specific activity 14 units/mg toward *N*-benzoyl-L-arginine ethyl ester at pH 6.5) contained 0.5 cysteine residue/mole of protein, as found by carboxymethylation with iodoacetic acid at pH 8.2 and analysis of the *S*-carboxymethylcysteine in the acid hydrolysate of the chromatographically separated protein. The cyanylation of the enzyme (0.05 m*M* in 0.1 *M* phosphate buffer, pH 7.4) with a fourfold molar excess of ^{14}C-NTCB was completed within 15 min, as determined spectrophotometrically. The enzyme lost 97% of its activity under these conditions. Chromatographic separation of the modified protein on Sephadex G-25 (Figure 2) showed that the enzyme contained 0.5 mol equiv of labeled carbon/mol of protein. Treatment of the cyanylated protein with a 50-fold molar excess of cysteine at pH 8.0 regenerated about 80% of the initial enzymatic activity. Gel filtration of this protein showed that it now contained only about 0.06 mol equiv of the labeled carbon/mol. This indicates that the cyanylation did indeed occur at the active cysteine residue of papain.

These results demonstrate that the NTCB is a remarkably mild and selective cyanylating agent for sulfhydryl compounds. Its pronounced reactivity is probably due to the displacement of a *p*-nitrothiophenolate, which is known to be a good leaving group for substitution by sulfur nucleophiles.[7,11] The characteristic optical absorption of the leaving group provides a convenient way to measure the kinetics of the cyanylation reaction and to differentiate between cysteine residues of varying reactivity in protein molecules. Introducing a radioactive *S*-cyano group by means of the readily available ^{14}C-NTCB enables quantitative evaluation of the extent of the modification and location of its site. The modification can be reversed by treatment with excess thiol, known to liberate cyanide from β-thiocyanoalanine residues.[12]

In previous experiments, we have tried another approach for converting cysteine residues into β-thiocyanoalanine residues, based on two steps

This indirect procedure worked well with model compounds such as glutathione, but with proteins such as papain and reduced ribonuclease, reaction 3 occurred in low yields, due to partial oxidation of cysteine to cystine residues. It appears that the intermediary mixed disulfide partly reacts with still-unreacted cysteine to form cystine and III. Similar oxidation of cysteine residues by DTNB has recently been reported.[13]

The selective conversion of cysteine residues into β-thiocyanoalanine residues offers two routes to specific

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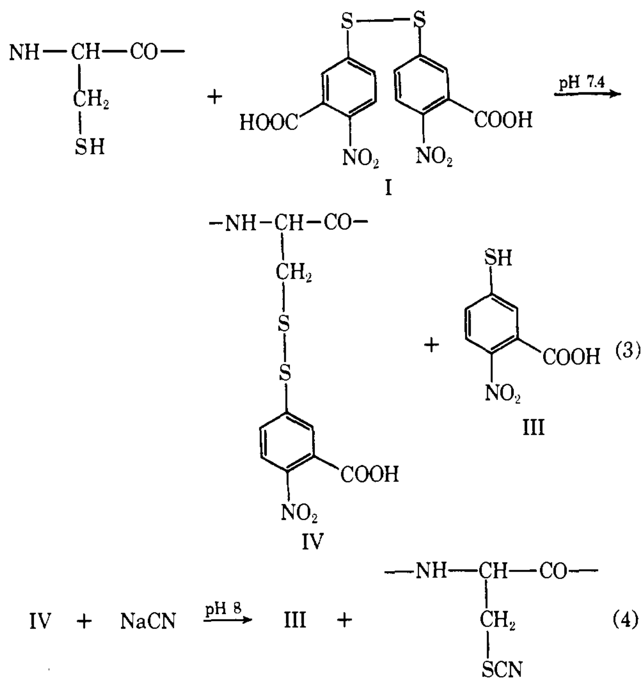

cleavage of peptide chains at the modified sites: (1) by cyclization to *N*-acyl-2-iminothiazolidine-4-carboxamide derivatives, followed by splitting of the *N*-acyl bond, as in the cyanide cleavage of cystine peptides;[5,6] and (2) *via* elimination of thiocyanate to form dehydroalanine residues.[14] Studies in both directions are now in progress. Again, S-cyanylation with ^{14}C-NTCB is advantageous for studying the cleavage reactions, in following either the appearance of radioactive 2-iminothiazolidine peptide derivatives or the eliminated radioactive thiocyanate.

Acknowledgment. We wish to thank Professor A. Nassner for helpful discussions. The work was supported by Grant No. AM-5098 from the U. S. Public Health Service.

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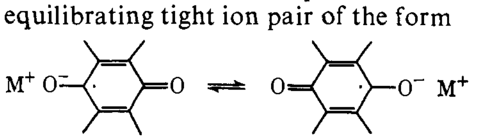
Detection of the Free Durosemiquinone Anion by Electron Nuclear Double Resonance Induced Electron Spin Resonance[1]

Sir:

In recent years there has been considerable interest in the structure and stability of the ion pair formed by the reaction of an alkali metal with duroquinone in ether solutions. The esr spectrum of the paramagnetic compound obtained can often be explained in terms of a rapidly equilibrating tight ion pair of the form



(1) Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Research Foundation of the State University of New York for support of this research.

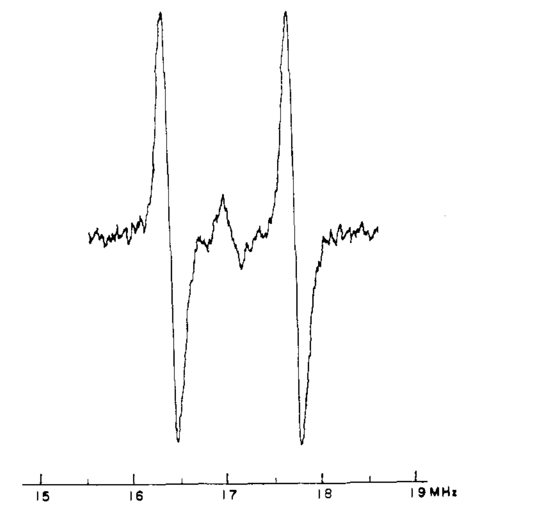


Figure 1. The high-frequency half of the endor spectrum of durosemiquinone at −78°.

the exact nature of the spectrum depending on the metal ion, the solvent, and their effects on the intramolecular ion-exchange rate. It has been suggested[2] and disputed[3] that for the anion formed from sodium or potassium in 1,2-dimethoxyethane (DME) solution, an additional solvent-separated ion pair or free ion must also be present to explain the entire temperature dependence of the esr spectrum.

While the low-temperature endor spectrum of the radical prepared with potassium in DME at low temperature shows the two pairs of lines corresponding to the two inequivalent sets of six protons observed in the esr spectrum of the radical under these conditions, careful investigation of the spectrum has shown an additional much weaker pair of lines with a coupling constant intermediate to those of the strong lines.[4a,b] The high-frequency half of a typical such endor spectrum is shown in Figure 1. Because the *g* value of the radical species responsible for the weak lines is not necessarily the same as that for the strong ones, the amplitude of the low-frequency magnetic field modulation has been adjusted to extend over the entire esr spectrum rather than just over the most intense line, as is normally done in endor experiments. This results in a substantial (>4) loss in the signal-to-noise ratio for the strong lines, but, we believe, more reliable intensity ratios.

To elucidate the structure of the radical responsible for the weak endor line, we have performed an endor-induced esr experiment.[5] The experiment is done by irradiating the sample at the frequency of the first-derivative maximum of an endor line and then sweeping the dc magnetic field through the esr resonance condition while monitoring the endor signal intensity. The resulting spectrum appears similar to that of the esr spectrum of the radical whose endor is being irradiated but exists only because of this rf irradiation. Figure 2 shows the endor-induced esr spectrum obtained at

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