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Rate Assay for Estimation of Thiol Affinity of Sulfhydryl-Reactive Agents: Estimation of SH-Reactivity

R. T. LOUIS-FERDINAND and G. C. FULLER

Abstract □ An interaction between cysteine SH groups and β-nitrostyrene was suggested from alterations in the ultraviolet spectrum of β-nitrostyrene following the addition of cysteine, but not serine. In the presence of β-nitrostyrene or phenylmercuric chloride, the rate of color development resulting from the reaction of cysteine and DTNB was reduced; increasing the concentration of β-nitrostyrene resulted in increased interference in the color development. Excess DTNB did not mitigate the influence of β-nitrostyrene on color development; however, excess cysteine was found to overcome the effects of the β-nitrostyrene on the development of color. The implication of this interference on single point SH content determinations is discussed. Application of inhibition of the cysteine DTNB reaction to estimate SH-reactivity of thiol-reactive agents is described.

Keyphrases □ Thiol affinity—sulfhydryl-reactive agents □ Sulfhydryl-thiol affinity—rate assay □ Cysteine–β-nitrostyrene interaction □ UV spectrophotometry—reaction monitoring

The inactivation of biochemical systems following exposure to sulfhydryl (SH)-reactive compounds and the reversal of the inhibition by thiols has been described earlier (1, 2). *In vitro* estimation of the relative affinities of SH-reactive agents for SH groups in such biological systems would be facilitated by the availability of a suitable model capable of reflecting variable SH-reactivity within a given series of compounds. In this investigation the ability of a series of SH-reactive agents to interfere with the rate of a disulfide-thiol reaction was examined, and employed to compare the SH-reactivity of certain mercurial and nitroolefinic derivatives.

EXPERIMENTAL

Reagents used were analytical grade or equivalent. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was used (Aldrich Chemical Co., Milwaukee, Wis.). Deionized water was used for all solutions. The β-nitrostyrene derivatives were prepared by the Department of Pharmaceutical Chemistry, University of Rhode Island.

The UV spectra of 2-ml. aliquots of β-nitrostyrene solutions (0.1 μmole/ml. of 0.1 *M* phosphate buffer pH 7.3) following the addition of 0.1 μmole/ml. solutions of cysteine or serine (0.1–0.2 ml.), were recorded using a spectrophotometer (Beckman DBG) and an accessory linear-log 25.4 cm. (10-in.) recorder. Reaction cells were maintained in a 30° thermostat-controlled spectrophotometer chamber during incubation and measurement periods.

Sulfhydryl reactivity was determined by following the rate of the color-forming reaction between cysteine and DTNB (3) in the presence and in the absence of the SH-reactive agent. The incubation media contained 7 ml. cysteine (0.25 m*M*), 10 ml. ethylenediaminetetraacetate (EDTA) (0.5 *M*) adjusted to pH 7.3 with potassium hydroxide, 5 ml. deionized water, and 6 ml. 0.1 *M* phosphate buffer (pH 7.3). Following the addition of ethanolic solutions of β-nitrostyrene or aqueous solutions of mercurials (0.05–0.15 ml.), the mixtures were incubated at 30° for 20 min. Twenty-five microliters of DTNB (4 mg./ml.) (prepared fresh daily in 0.1 *M* phosphate buffer pH 7.3) were pipeted into the sample cell to start color development. The rate of absorbance increase at 412 mμ was estimated from straight line portions of the recordings, which were commenced 5 sec. after the addition of DTNB.

RESULTS

Effect of Cysteine on the Ultraviolet Spectrum of β-Nitrostyrene—The effect of the addition of cysteine solutions on the UV spectrum of β-nitrostyrene is shown in Fig. 1. The alterations observed include a decrease in the absorption at 300 mμ, an increase in the absorption at 250 mμ, and a progressive shift in the absorption maximum at 230 mμ to shorter wavelengths. Isosbestic points in the family of spectra can be seen near 250 and 263 mμ. The result of similar additions of serine to β-nitrostyrene solutions led to no qualitative spectral changes. The quantitative alterations seen with serine were no greater than those to be expected from dilution of β-nitrostyrene solutions. These data are suggestive of an interaction between β-nitrostyrene and SH groups of cysteine.

Sulfhydryl Assay—Development of colored reaction product was followed by measuring absorption at 412 mμ, after the addition of DTNB to a solution of cysteine. Enhanced cysteine and product

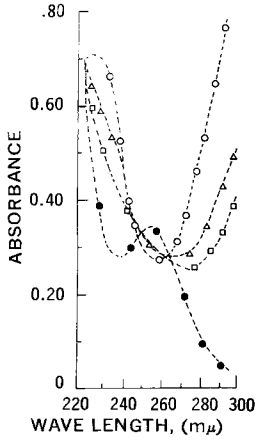

Figure 1—*Effect of cysteine on the UV spectra of β-nitrostyrene solutions. Key: UV spectrum of β-nitrostyrene solution (BNS) 0.1 μmole/ml. (—○—○—); BNS plus 0.10 μmole cysteine (—△—△—); (BNS) plus 0.20 μmole cysteine (—□—□—); BNS plus 2.2 μmole cysteine (—●—●—).*

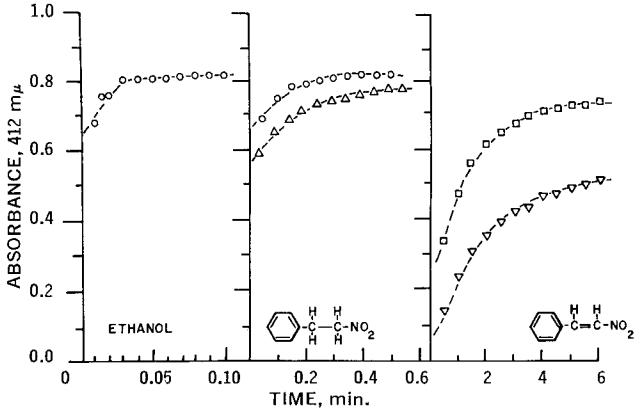

Figure 2—*Effect of β-nitrostyrene and 1-phenyl,2-nitroethane on the color development due to the reaction of cysteine with 5,5′-dithiobis (2-nitrobenzoic acid). Cysteine concentration was 0.075 mM. Key: from left to right: ethanol 0.2 ml./ml. reaction medium (—○—○—); 1-phenyl,2-nitroethane 0.24 mM (○—○), 0.53 mM (—△—△—); β-nitrostyrene 0.13 mM (—□—□—), 0.36 mM (—▽—▽—).*

stability was found when EDTA was included in the reaction medium. The absorbance obtained following the reaction of DTNB with cysteine in the presence of 0.18 *M* EDTA was found to be linearly related to cysteine concentration. The molar absorptivity for the reaction product, 13,400, was similar to that reported by others (3).

The appearance of colored reaction products as followed by absorption at 412 mμ following the addition of DTNB to a solution of cysteine, is shown in Fig. 2. The absorbance approaches a maximum value within 0.03 min. after commencement of the measurement period. In the presence of 0.13 m*M* β-nitrostyrene, the absorbance increases more gradually. An excess of DTNB does not increase the rate at which the absorbance changes in the presence of β-nitrostyrene. When cysteine is omitted from the reaction medium, the addition of DTNB results in an absorption of 0.012 absorbance units with 0.26 m*M* β-nitrostyrene in the incubation medium with no further change in the absorbance. The interference with the progress of the reaction increases with increasing β-nitrostyrene concentration. In the presence of an excess of cysteine—*viz.*, 3 m*M*, the effect of β-nitrostyrene concentrations up to 0.49 m*M* are not discernible.

The effect of certain nitroolefin derivatives and phenylmercuric chloride on the reactivity of cysteine solutions with DTNB is shown in Table I. At this cysteine concentration (0.017 m*M*) the initial slope of the absorbance-time recording decreases with increasing β-nitrostyrene concentration. The presence of 3.6 parts per million phenylmercuric chloride results in a greater degree of interference with the development of absorbance at 412 mμ than obtained with similar concentrations of β-nitrostyrene. The reciprocal slope of the absorbance-time plot was used to simplify comparison of inhibitors.

Table I—Comparison of the Effect of Certain Nitroolefins and Phenylmercuric Chloride on the Development of Color Due to the Reaction[a] Between Cysteine and 5,5′-Dithiobis (2-Nitrobenzoic Acid)

Agent	Concn., m*M*	Reciprocal Slope, min./OD unit
4-Propionoxy β-nitrostyrene	0.13	10.1
4-Propionoxy β-nitrostyrene	0.43	17.9
β-Nitrostyrene	0.17	12.3
β-Nitrostyrene	0.42	23.8
β-Nitrostyrene	0.88	45.5
Phenylmercuric chloride	0.011	143.0

[a] Concentrations of reagents used were: cysteine 0.017 m*M*; phosphate pH 7.3 buffer 0.026 *M*; 5,5′-dithiobis (2-nitrobenzoic acid) 0.084 m*M*.

From Table I it is seen that the reciprocal slope in the presence of phenylmercuric chloride is approximately 10 times greater than that in the presence of β-nitrostyrene. Saturation of the α-β-double bond of β-nitrostyrene results in a product in which the ability to interfere with the DTNB-cysteine reaction is considerably diminished (Fig. 2).

The interference by β-nitrostyrene with the sulfhydryl assay was determined in the presence and absence of serine in order to estimate the specificity of β-nitrostyrene for thiol compounds under the conditions of this assay. The rate of color development resulting from the reaction between cysteine and DTNB is essentially the same in the presence or absence of serine.

The addition of DTNB to aliquots removed from similar incubation media from which cysteine had been omitted results in negligible absorption at 412 mμ.

DISCUSSION

These results provide evidence for an interaction between cysteine and BNS which interferes with the progress of the reaction in the colorimetric DTNB-SH assay procedure described initially as a single point determination (3). Such interference may result in the underestimation of SH concentration under conditions where the cysteine-DTNB reaction has not gone to completion. Color development due to the reaction between cysteine and DTNB is progressively delayed with increasing BNS concentration. Similar results are obtained when mercaptoethanol is used in place of cysteine. Excess cysteine abolishes the effect of BNS on the color development while excess DTNB is without effect. Thus it is apparent that the cysteine concentration is the limiting factor in the progress of the color development under the described assay conditions.

The specific nature of the interaction is suggested by the absence of an interaction between BNS and a structural analog of cysteine (serine). Further, the SH reactivity of BNS is considerably reduced by saturation of the α-β-nitroolefinic bond. These results suggest that the interaction between cysteine and BNS is specific for SH groups and the α-β-nitroolefinic bond of BNS.

The interference with the cysteine-DTNB reaction reflected by the observed delay in color development was utilized in this investigation as a model system to compare the relative reactivity of a group of SH-reactive compounds. Similar effects on the DTNB-SH reaction have been reported by Gabay *et al.* (4) who have reported that the color development of solutions containing non-protein sulfhydryl material (following addition of DTNB) is delayed in the presence of bovine serum albumin. These data also indicate the applicability of the rate procedure described here for the estimation of the affinity of thiol-reactive substances for SH groups. Correlative studies are currently in progress in which the model system

described in this report is utilized for the screening of molluscicide activity.

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Peyote Alkaloids IX: Identification and Synthesis of 3-Demethylmescaline, a Plausible Intermediate in the Biosynthesis of the Cactus Alkaloids

GOVIND J. KAPADIA, Y. N. VAISHNAV, and M. B. E. FAYEZ*

Abstract □ The presence of 3-demethylmescaline in the alkaloidal mixture of peyote was proven by GLC-mass spectrometry through direct comparison. A new synthesis of this compound was realized by elaborating the ethylamine side chain onto the appropriately substituted benzene residue (with benzyl-etherification of the phenol group). The sequence involved transformation of the acid chloride successively into the diazoketone, the acid amide, and the ethylamine. A discussion is given of the possible role of 3-demethylmescaline and its congeners in the biosynthesis of peyote alkaloids.

Keyphrases □ Peyote alkaloids □ 3-Demethylmescaline—isolation, identification □ Synthesis, 3-demethylmescaline—structure confirmation □ GLC—separation, identity □ Mass spectroscopy—identity, structure

The role of partially methylated phenolic intermediates in the biosynthesis of the phenethylamine and tetrahydroisoquinoline alkaloids of peyote (*Lophophora williamsii*) may well be anticipated in view of the proven (1) involvement of the hydroxylic rather than the methoxylic phenethylamines. Such partially methylated intermediates of the trioxygenated phenethylamine may be envisaged to transform *in vivo* into products such as mescaline (the principal alkaloid in the plant) by *O*-methylation and also into several possible tetrahydroisoquinolines by cyclization. A consideration of the nature and distribution of substituents in the latter type of peyote alkaloids, where several of them carry a hydroxyl group on C-8, suggests that a likely precursor is specifically 3-hydroxy-4,5-dimethoxy-β-phenethylamine (3-demethylmescaline) (I). The authors (2) have earlier identified the *N*-formyl and *N*-acetyl derivatives of I in the nonbasic fraction of peyote, a fact which prompted a search for this relatively simple alkaloid in the basic fraction of the plant.

Inspection by GLC of the phenolic-basic fraction obtained from peyote revealed the presence of no less than ten products which include the previously reported (3) hordenine, pellotine, anhalonidine, and anhalamine. The presence[1] of I in the mixture was demonstrated unequivocally by direct comparison in the gas chromatograph and from a consideration of its mass spectrum. The latter contained ions at *m/e* 197 (M^+), 168 (100%, loss of CH_2NH), 167 (hydroxydimethoxybenzyl cation), 153 (loss of CH_3 from ion *m/e* 168), 137 (loss of CH_3O from ion *m/e* 168), 125 (loss of CO from ion *m/e* 153), and 123 (loss of CH_2O from ion *m/e* 153).

In order to obtain experimental evidence for the role of I in the biogenetic pattern of peyote alkaloids, it was necessary to secure a convenient method for its synthesis.[2] Several approaches were tried all starting with, methyl gallate followed by partial methylation to the 3,4-dimethyl ether (II). The complexities arising from the unprotected phenol group rendered abortive the route (6) proceeding by lithium aluminum hydride reduction of the methyl ester of II to give the benzyl alcohol III. The latter was intended to lead successively to the corresponding benzyl chloride and cyanide derivatives and ultimately to I by reduction. For the same reason the diazoketone IV—obtained from II *via* the corresponding *O*-carbomethoxy derivative and acid chloride—failed to give an acid amide upon treatment with silver nitrate in ammoniacal solution, in which the phenol group was doubtless regenerated. These difficulties were overcome only by initial benzyl etherification of the phenol group in II. This product (V) was trans-

[1] After the present work was completed, Agurell and Lundström (4) reported the detection of I in peyote using a similar technique. However, the authors identification of 3-demethylmescaline herein reported has previously been presented before the Organic Chemistry Discussion Group, National Institutes of Health, on October 15, 1968.

[2] A different synthesis of this compound has much earlier been reported by Späth and Röder (5). However, due to the difficulties met in preparing the starting material (II) and in purifying the aldehyde (R = CHO, R_1 = $COOC_2H_5$) intermediate in this synthesis in addition to the low overall yield of the final product, it was decided that an alternative route was desirable.